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CANCER RESEARCH

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Nucleic Acids in Relation to Tissue Growth: *A Review*

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The period that has elapsed since the review of this subject in 1944 (18) has brought an ever increasing flow of scientific papers dealing with nucleic acids and nucleoproteins, but, although the information available on this subject has increased abundantly, we are still unable to explain the precise biological function of the nucleic acids. While it is clear that they play an important and probably fundamental part in the processes of cellular growth and division and in the synthesis of proteins, their exact mode of action has not yet been satisfactorily explained. The general aspects of nucleoprotein biochemistry have been adequately reviewed elsewhere of recent years (9, 10, 26, 42), and an attempt will be made here to deal only with a few aspects which relate specifically to the problem of growth.

One of the most interesting recent developments, of fundamental importance in itself but also with far-reaching implications, relates to the desoxyribonucleic acid (DNA) content of the individual cell nucleus. In 1948 Boivin, Vendrely and Vendrely (3, 48) published figures for the DNA content of the individual cell nucleus of vertebrates, showing that for a given species the amount was constant for the somatic cells of different tissues and was approximately double that found in the haploid cells (spermatozoa). On the other hand, there might be considerable variation from species to species, fish and birds having in general a lower DNA content in the nucleus than mammals, which tended to give figures of the order of 5 to 6×10^{-6} μg. per nucleus (51). The general conclusions reached by the Vendrelys were confirmed by Mirsky and Ris (34), who examined a wide range of vertebrates with nuclear DNA contents ranging from 1.97×10^{-6} μg. (shad) to 15.0×10^{-6} μg. (frog). Similar results have been obtained recently by other authors, whose results are summarized in Table 1.

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Since conventional genetic theory assumes that the chromosome complement is constant for all the somatic cells of a given species, the results for the chemical analyses of isolated cell nuclei are scarcely surprising provided we accept the view that DNA is an integral and constant component of the chromosomes in the resting state. Cytological opinion on this point differs, some authorities maintaining that a given chromosome may be starved of DNA or overloaded, according to circumstances. However this may be, it is clear that the situation will be complicated in a tissue containing many dividing cells, since at some as yet undetermined stage in the process of mitotic division, the DNA content of the cell must be doubled. A further complicating factor is the frequent occurrence of various degrees of polyploidy in certain tissues, and this may account for the surprisingly high values for the DNA content of the liver cell nucleus of the rat (15, 40), since tetraploid and octoploid nuclei are regularly found in the rodent liver (29). In the different tissues of the plant Tradescantia, however, Schrader and Leuchtenberger (44) have found widely varying amounts of DNA which they attribute not so much to polyploidy as to the different degrees of polyteny which are present in the various tissues; but their conclusions have been criticized by Mirsky (41).

If the constancy of the DNA in the cell nucleus is in fact related to the chromosome content, it would be expected, as has indeed been found, that the sperm cells containing the haploid number of chromosomes would contain half the amount of DNA found in the somatic cells (Table 1). But it would also be expected that the egg would have

the same DNA content as the sperm. This view has been put to the test by Vendrely and Vendrely (49), who found a very much larger amount of DNA in the eggs of Arbacia than in the sperm. This discrepancy has not yet been satisfactorily explained, but it is in agreement with the observation of Schmidt, Hecht, and Thannhauser (43) that the DNA in Arbacia sperm is 3 per cent of that in the egg.

It has already been noted (8) that fasting causes no change in the total DNA *content* of the rat liver, although ribonucleic acid (RNA) and phospholipid are considerably reduced; and it has now been demonstrated that, as might well be expected, the amount of DNA in isolated liver nuclei is the same in fasted as in fed animals (15). Similar observations on rats kept for long periods on a protein-free diet have been made by Mandel and Jacob and Mandel (31, 32) who found no change in the number of nuclei or in the total DNA content of the liver.

It must be emphasized that the results shown in Table 1 have been obtained from small numbers of specimens (in some cases from single specimens) of each type of tissue. In an attempt to cover a wider range, Davidson, Leslie, and White (14) examined eighteen specimens of normal human bone marrow from sixteen individuals and found a mean value for the DNA phosphorus content of the nucleus of 8.54×10^{-7} μg. (i.e., approx.

TABLE 1

DESOXYRIBONUCLEIC ACID CONTENT OF SINGLE NUCLEI (μg. $\times 10^6$)

Species	Liver	Erythrocyte	Kidney	Thymus	Pancreas	Sperm	Reference
Cattle	6.4		5.9	6.4	6.9		3, 48, 50
"	6.2, 8.4		6.3, 6.8	7.2		2.8	34
Calf	6.5						41
Pig	5.0		5.2				3, 48, 50
Guinea pig	5.9						3, 48, 50
Dog	5.0, 5.5		5.3				50
Man	5.8, 5.9, 6.3						50
"	10.0		7.5			3.4, 4.4	14
Rabbit	7.2						15
"	5.3						50
"	7–9		7–9				30
Rat	10.1–14.0						40
"	7–9		7–9				30
"	9.2–11.2						15
"	10.7		7.3				12
Mouse	7–9		7–9				30
"	6.0		5.0				50
Horse	5.8						50
Sheep	5.4, 6.1						50
Frog	15.7	15.0					34, 41
Green turtle	5.1	5.3					34
Fowl	2.4	2.3				1.26	34, 41
"	2.3	2.4	2.2		2.3		12
18-day chick embryo*	2.4						12
Duck	2.1	2.3					50
Goose		1.9					51
Turkey		1.9					51
Pheasant		1.7					51
Pigeon		2.0					51
Sparrow		1.9					51
Carp	2.8, 3.2						50, 51
"	3.3	3.5				1.64	34, 41
Shad	2.0	1.97				0.91	34, 41
Trout		5.79				2.67	34
"		4.8					51
Roach		7.3				3.7	34
Yellow tail	2.1						41
Red hind	2.1						41
Houndfish	2.2						41
Yellow grunt	1.2						41
Dusky shark	5.5						41
Tench		1.8					51
Eel		1.9					51
Pike		1.7					51

* Figures for the chick embryo (12) are as follows:
14-day: Brain, 2.2
Liver, 2.3
18-day: Heart, 2.2
Liver, 2.4
Muscle, 2.4

8.5×10^{-6} μg. DNA) with a standard error of 2.89. The observed range, however, was somewhat wider (4.0–15.0) than might have been expected, even allowing for the unavoidable inaccuracies involved in the method of cell counting which was employed; but values below 6 were very rare.

Observations on the DNA content of individual tumor cell nuclei are few. Mark and Ris (33) found the same DNA content in hepatoma and in cholangioma as in normal rat liver nuclei; and Cunningham, Griffin, and Luck (7) found a constant value of 6×10^{-6} μg. DNA per nucleus in normal rat liver and in the livers of rats showing precancerous changes as the result of feeding acetylaminofluorene or 3′-methyl-4-dimethylaminoazobenzene. Price *et al.* (40) found somewhat higher values (range 10–14 $\times 10^{-6}$ μg. per nucleus) in nuclei from normal rat liver and from the livers of animals treated with aminoazo dyes. Moreover, in a comparison of bone marrow cells from normal human subjects with those from patients with leukemia, no significant difference was found between the mean values of the DNA content in the normal and leukemic series (14), although the DNA content of the marrow cell nuclei was significantly raised in cases of pernicious anemia.

On the other hand, Klein, Kurnick, and Klein (30) found that mouse ascites tumor cells had a DNA content (20×10^{-6} μg. per nucleus) which was much higher than that for normal mouse liver and kidney cells, while Davidson and McIndoe (15) have found a higher value (5.1×10^{-6} μg. DNA) in GRCH 15 tumor[1] cell nuclei than in normal fowl nuclei.

In relation to the constancy in the amount of DNA in the cell nucleus, it is interesting to note that recent work with isotopes has emphasized the stability of this material. Davidson and Raymond (16) administered labeled ammonium salts to rats and pigeons and found little or no incorporation of N^{15} into the DNA fraction of the liver tissue, but their experiments were carried out with very low concentrations of the isotope. More recently, Furst, Roll, and Brown (20) have confirmed these results, working on a much larger scale, and have shown that isotope-labeled dietary adenine is rapidly incorporated into the purines of the RNA of rat liver, whereas its incorporation into the purines of the DNA fraction is only about 1 per cent as extensive, and they consider it probable that all the isotope incorporated into the DNA is introduced in the process of formation of new nuclei. DNA may thus be an exception to the general concept of a rapid dynamic equilibrium of body constituents, and its skeleton may not be at all in dynamic equilibrium with the other constituents of the cells. Experiments with P^{32} (4, 8, 16, 25) have also demonstrated that the turnover of RNA is much greater than that of DNA, although the inert nature of DNA is less evident with P^{32} than with N^{15}; but with both isotopes it can be shown that the turnover of DNA is very greatly increased when mitotic division is induced after partial hepatectomy (4, 16, 20).

If this hypothesis of the constancy of DNA in the cell nucleus is in fact correct, the implications are indeed far-reaching, for we have here a chemical unit which can be used in two ways, either as a measure of cell multiplication or as a unit of reference by which the changing composition of the cell may be expressed during the processes of growth, differentiation, and regeneration (11).

It is clear that, if the DNA content of the cell nucleus is constant, an estimation of the amount of DNA in a sample of tissue will enable us at once to calculate the number of cells present, and it will provide us with an index for following the process of growth. The application of this principle can be conveniently illustrated by a consideration of the growth of explanted tissues *in vitro*. The estimation of the growth of cells in tissue culture is a matter of some difficulty which is complicated by the different interpretations which various authors have put upon the word "growth," and these problems have been reviewed by Cunningham and Kirk (6). We ourselves have adopted the suggestion made by Willmer (52) that the growth of a culture might be measured by the increase in its nucleoprotein content as determined chemically (17, 19), and we have extended it to the estimation of ribonucleic acid phosphorus (RNA P) and desoxyribonucleic acid phosphorus (DNA P) separately (13). Our experiments along these lines with chick embryo heart explants had convinced us that in the early stages of growth the RNA P rose more sharply than the DNA P (an observation which has since been confirmed by Signorotti, Hull, and Kirk [27, 45]) and that real growth could not be said to have occurred without a rise in DNA P. If we assume that the DNA P content of the fowl cell nucleus lies between 2.3 and 2.6×10^{-7} μg. (12)—the actual figure is immaterial to the argument—it is possible to convert the DNA P content of the cultures into a figure for the number of cells present.

The second aspect of this problem concerns the use of DNA as a unit of reference by which we may reveal the changing composition of cells in

[1] The GRCH 15 tumor is a nonfiltrable fowl sarcoma first produced by Dr. P. R. Peacock of the Glasgow Royal Cancer Hospital in 1939 by 1:2:5:6-dibenzanthracene and maintained since by serial transplantation.

which the relative proportions of the different constituents are continually varying. The value of this principle may be illustrated by referring again to cultures of fresh explants of chick heart embryo *in vitro* in which the changing amounts of protein nitrogen, acid-soluble phosphorus, lipid phosphorus, and RNA P have been referred to the DNA P content. In Figure 1 it is possible to compare the changes in cell composition which correspond to each stage of cell multiplication in chick heart explants cultured *in vitro*. The cell number, which has been calculated on the basis

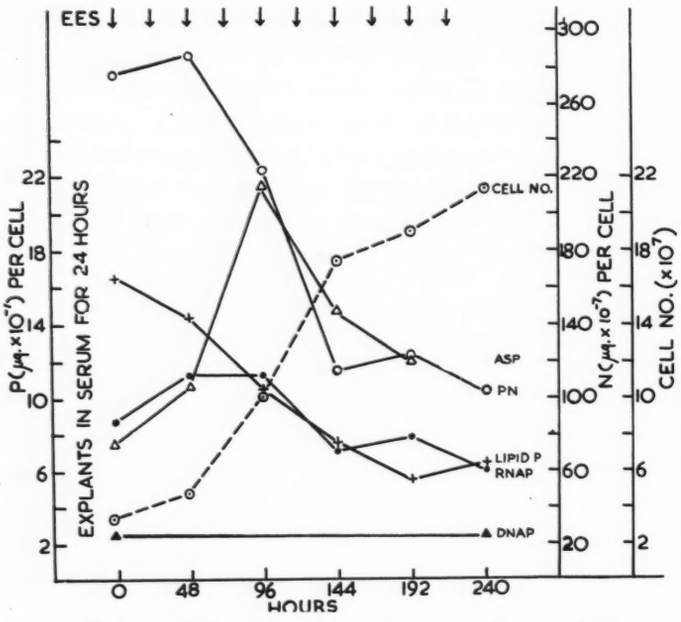

FIG. 1.—Changes in cell number and cell composition of chick heart explants growing *in vitro*. Results have been calculated on the basis that the fowl nucleus contains 2.35×10^{-7} μg. DNA P. Embryo extract and serum medium (EES) replaced every 24 hours throughout growth. ASP = acid-soluble phosphorus (no result at 240 hours); PN = protein nitrogen; RNA P = ribonucleic acid phosphorus; DNA P = desoxyribonucleic acid phosphorus.

that the DNA P content of fowl nuclei is 2.35×10^{-7} μg., increases most rapidly between 48 and 144 hours, nearly doubling in each 48-hour interval. The initial cell composition, measured after the explants have been in contact with diluted fowl serum for 24 hours, shows, on the average, 2–3 times as much protein and phospholipid per cell as the culture cells at the end of 144 hours' growth. (Throughout the run the growth-promoting medium was renewed every 24 hours.) The decrease in the content of two major constituents of the cell suggests that a decrease in average cell size must also occur. This view finds confirmation in a detailed study of the growth of chick heart explants carried out by Tompkins, Cunningham, and Kirk (47). These authors were able to show from measurements of cell cross-sectional area that the average mass per cell in the peripheral region was 2–4 times that of cells in the interior of the explant; and that, when mitotic activity in the peripheral region declined after 72 hours, the average cell mass in that region also declined. It seems likely, therefore, that the high protein and phospholipid content of the cells in the early stage of growth is a reflection of the increase in cell size prior to rapid cell multiplication. It is also clear from these results that a high cell division rate is accompanied by a peak in the content of acid-soluble phosphorus and RNA P in the cells. (After 144 hours the explants continue to grow slowly and remain fairly constant in composition.)

In biochemical studies on embryonic growth, the changing cell composition can be followed in the same way, thus avoiding the serious difficulties in interpretation which arise when results are expressed as concentrations of fresh or dry weights. By relating the various components to a unit of DNA phosphorus, we have been able to show, for example, that the protein content of the cells of chick embryonic brain, liver, and skeletal muscle increases steeply over certain stages of development (11). Confirmation of these results for chick embryonic brain can be obtained from the work of Bieth, Mandel, and Stoll (1, 2). Where these authors record a tenfold increase in the content of protein per brain (1), calculation of the protein per cell on the basis of the DNA content shows only a fourfold increase over the same embryonic period. The results recorded in this paper confirm the striking increase in the phospholipid content of the brain cells, which we have found to be a characteristic of that tissue during embryonic growth of the chick (12).

Where other authors have expressed their results in relation to the DNA P content of the tissue, it is possible to make similar interpretations. For example, the nearly threefold increase in the ratio of RNA P/DNA P, which Geschwind and Li (21) found in rat liver during fetal development and early growth, can be interpreted as an increase in the RNA P content of the liver cells. Their figures show that during the same time the concentration of RNA P in the liver as a whole dropped by one-half. Novikoff and Potter (38) in their study of nucleic acids in the developing chick embryo based their interpretations on the fact that the concentrations of both RNA and DNA reach a peak on the fifteenth day of development. However, if the relative amounts of RNA per cell are obtained from the ratio RNA/DNA, the average RNA content of the cells appears to rise to a maximum on about the eleventh day. Any inter-

pretation is difficult in such a complex system of development as the whole embryo, but one based on the ratio RNA/DNA has the advantage of being independent of the changing proportions of water and other constituents in the embryonic tissues.

So far, we have been discussing chemical changes recorded during embryonic growth as events occurring during definite phases of development. However, relating them in this way to embryonic age tends to hide the fundamental chemical relationships which must exist between the various constituents of dividing cells. As a means of showing how increases in one cell constituent are quantitatively related to increases in others, we (11) have made use of the simple allometric relationship $y = bx^k$ originally applied by Huxley (28) to morphological development, and by Teissier (46) and Needham (36, 37) to biochemical development. In these earlier biochemical studies, the growth rate of a component was measured in relation to the growth rate of the whole organism or tissue, expressed in terms of wet or dry weight. The weakness of this method lies in the fact that proportions of other cell constituents are constantly changing during growth and that the component being investigated is itself one of the variables which determines the total weight. If, instead, the components are related on the double logarithmic grid to the DNA of the tissue, we can compare their rates of accumulation in relation to one another and to the increasing cell number as expressed in the amounts of DNA.

Recent results (12) showing the relative growth rates of various cell constituents in embryonic chick brain, heart, and liver, reveal that the accumulation of lipid phosphorus, RNA P, and protein nitrogen follows a similar pattern in all three tissues. In brain, however, the relative growth rate of phospholipid does not correspond so closely to the other growth ratios, but is consistently higher in both phases of development as can be seen in the accompanying graph (Fig. 2). Assuming that the DNA P content of brain nuclei remains constant throughout embryonic development, as we have found to be the case for 14- and 19-day-old embryos (12), the cells must be rapidly increasing in content and size in the first phase, while in the second the phospholipid continues to increase at a greater rate than the other components. If one compares the increases in DNA P per organ in brain, heart, and liver, it appears that there is only a fourfold increase in the number of brain cells during the period in which heart and liver cells multiply 30 and 40 times, respectively. It is clear from these results alone that increase in cell size is making a larger contribution to growth in brain than is the case in heart and liver. The changes in the relative growth rates of all four components in brain occur at a point on the DNA P scale reached about the fourteenth to fifteenth day of development. This is also true of the liver, and it confirms earlier observations of a critical phase in embryonic development at this stage (35).

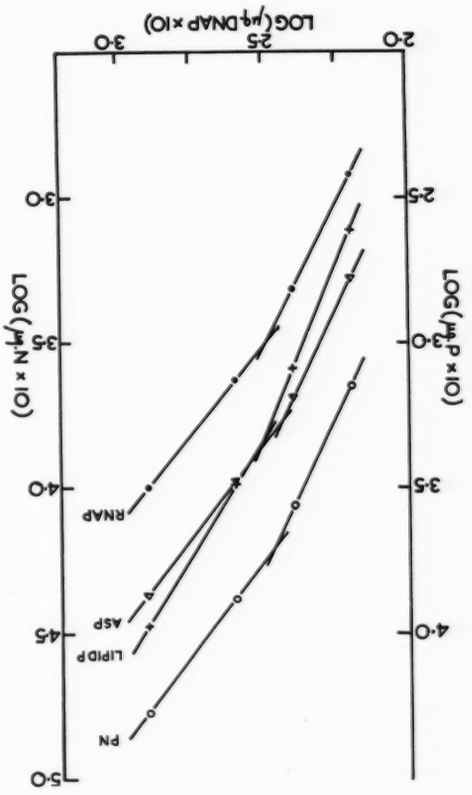

FIG. 2.—Growth rates of constituents of chick embryo brain relative to that of DNA P. The lines were obtained by plotting results on large-scale graphs, and the points as actually determined are not shown in this figure. ASP, PN, RNA P, and DNA P as in Figure 1.

Greenstein (22) has stressed the fact that all cancerous tissues show a strong resemblance to one another in their chemical pattern, and that the more autonomous they are "the more do they deviate from the chemical pattern of their tissues of origin, and the closer do they approach an apparently functionally undifferentiated type of tissue presumably common to all." Consequently, it is particularly important to have some knowledge of the chemical events which occur during the transformation of normal into neoplastic tissues and so bring about this convergence of chemical type. A number of interesting studies along these lines have been in progress for some time, and, where these include determinations of DNA which

can be related to amounts of other cell constituents, it is possible to see how changes based on concentrations differ from these expressed as ratios to this nuclear constituent.

Griffin, Nye, Noda, and Luck (24), recording the percentage composition of rat liver tissue from animals fed 3′-methyl-4-dimethylaminoazobenzene, showed that the nitrogen and RNA concentrations fell but slightly during hepatoma development, while the DNA concentration increased steadily and the riboflavin concentration decreased to about half its original value. Similar findings are recorded by Price, Miller, Miller, and Weber (39), who compared the effects of azo dyes of different carcinogenic potency on the composition of fractions of rat liver tissue obtained by differential centrifugation, and found little change in the protein concentration of the whole tissue with increasing carcinogenicity, although the levels of protein, RNA, and DNA were greatly increased in the nuclear fraction. At the same time the most active carcinogen lowered the levels of protein, RNA, and riboflavin in the large and small granule fractions to nearly those levels found in liver tumors.

A rather different picture emerges if we accept

TABLE 2

EFFECT OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE ON THE COMPOSITION OF RAT LIVERS*

	Control basal diet	Weeks for which diet containing dye was fed				Hepatomas
		2	4	6	8	
	mg. per milligram DNA					
Nitrogen	9.48	6.24	5.61	5.10	4.88	2.70
Nonprotein nitrogen	0.68	0.46	0.48	0.42	0.46	
Phosphorus	1.05	0.80	0.72	0.63	0.65	0.36
Nonprotein phosphorus	0.30	0.25	0.24	0.22	0.21	
Ribonucleoprotein as ribose	0.56	0.31	0.33	0.29	0.25	0.18
Riboflavin (μg.)	9.3	5.0	4.0	2.8	2.5	

* Calculated from data of Griffin, Nye, Noda, and Luck (24).

TABLE 3

EFFECT OF AMINOAZO DYES ON THE COMPOSITION OF RAT LIVER FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES*

	Control without dye	AB	4′-Me-DAB	DAB	3′-Me-DAB
	mg. per milligram DNA				
Protein in:					
whole liver homogenate	65.5	66.5	61.0	48.6	25.65
nuclear fraction	8.3	6.67	8.1	7.75	8.1
large cytoplasmic granules	21.5	21.4	19.4	12.54	3.88
small cytoplasmic granules	9.1	10.19	9.45	6.34	2.88
supernatant fluid	25 3	25.20	23.5	19.6	10.00
Ribonucleic acid in:					
whole liver homogenate	2.93	2.67	2.31	2.15	1.12
nuclear fraction	0.24	0.23	0.19	0.16	0.29
large cytoplasmic granules	0.95	0.84	0.74	0.54	0.17
small cytoplasmic granules	0.96	0.86	0.71	0.46	0.23
supernatant fluid	0.66	0.69	0.58	0.52	0.36
Riboflavin (μg.) whole homogenate	5.3	5.3	6.1	3.1	1.4
nuclear fraction	0.32	0.43	0.60	0.32	0.42

AB = 4-aminoazobenzene (non-carcinogenic)
4′-Me-DAB = 4′-methyl-4-dimethylaminoazobenzene (weakly carcinogenic)
DAB = 4-dimethylaminoazobenzene (moderately strongly carcinogenic)
3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene (strongly carcinogenic)

* Calculated from the mean values of the figures given by Price, Miller, Miller, and Weber (39).

TABLE 4

EFFECT OF 2-ACETYLAMINOFLUORENE ON THE COMPOSITION OF RAT LIVERS*

	Control basal diet	Weeks for which diet containing carcinogen was fed						24 weeks +4-week basal diet	
		2	4	6	14	20	24	Liver	Tumor
	mg. per milligram DNA								
Nitrogen	9.3	9.9	8.0	7.3	9.7	11.8	10.1	8.4	6.2
Phosphorus	1.1	1.3	0.9	0.9	1.1	1.4	1.3	1.1	0.7
RNA as ribose	0.51	0.54	0.32	0.37	0.41	0.60	0.44	0.63	0.38
Riboflavin (μg.)	9.3		6.6		6.8		7.5	5.6	1.0

* Calculated from data of Griffin, Cook, and Cunningham (23).

the conclusions of various authors (7, 33, 40) that the DNA content of the rat liver cell nucleus is unchanged during carcinogenesis, for we may then calculate the change in cell composition in relation to the unchanging DNA (Tables 2, 3, and 4). Table 2 shows that during carcinogenesis a steady but very pronounced fall in the protein content of the cell occurs, accompanied by a corresponding but less marked decrease in RNA and riboflavin. The fall in the protein content, which is confirmed in Table 3, affects mainly the large cytoplasmic granules, although the small granules and non-granular cytoplasmic material are also involved. On the other hand, the protein content of the nucleus shows very little change. It also appears that the RNA and riboflavin decrease in the cell but not in the nucleus. In this way, it becomes clear that, in rat liver tissue at least, carcinogenesis affects chiefly the cytoplasm and is accompanied by a dramatic fall in its protein content, especially in the large granules.

These results have been confined to the effect of one type of carcinogen on rat liver. Griffin, Cook, and Cunningham (23), however, have compared the chemical changes during the induction of rat liver tumors by acetylaminofluorene with those produced by 3′-methyl-4-dimethylaminoazobenzene, and have come to the conclusion that the that the mode of action of these two carcinogens is different. The interpretation of their findings is not substantially changed when their results are related to a unit of DNA (Table 4), but results expressed in this way can be correlated with histological observations (5) in that the average nitrogen and RNA content per cell rise during the time when the cytoplasmic volume within the cells of the resulting liver nodules is distinctly greater than normal. The final effect of both carcinogens is similar, for the liver tumors produced in each case have lower contents of nitrogen, RNA, and riboflavin per cell than are found in the normal liver or in the liver in which the tumor has developed. Our only observation on a human tumor at present shows that in a myoblastoma the cells contain one-sixth of the amount of protein found in the cells of the nearby muscle. If this lowered protein content of the cells is a typical and persisting feature of tumor tissue, as seems likely, it provides an interesting contrast to our earlier suggestion (11) that normal embryonic growth and differentiation is characterized by an increasing protein content of the dividing cells.

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REFERENCES

1. BIETH, R., and MANDEL, P. Développement biochimique du cerveau de l'embryon de poulet. II. Les lipides. Bull. Soc. chim. biol., **32**:109–15, 1950.
2. BIETH, R.; MANDEL, P.; and STOLL, R. Évolution biochimique du cerveau de l'embryon de poulet. Les acides nucléiques. Compt. rend. Soc. de Biol., **142**:1020, 1948.
3. BOIVIN, A.; VENDRELY, R.; and VENDRELY, C. Biochimie de l'hérédité. L'acide désoxyribonucléique du noyau cellulaire, dépositaire des caractères héréditaires; arguments d'ordre analytique. Compt. rend. Acad. d. sc., **226**:1061–63, 1948.
4. BRUES, A. M.; TRACY, M. M.; and COHN, W. E. Nucleic Acids of Rat Liver and Hepatoma; Their Metabolic Turnover in Relation to Growth. J. Biol. Chem., **155**: 619–33, 1944.
5. COX, A. J.; WILSON, R. H.; and DEEDS, F. The Carcinogenic Activity of 2-Acetylaminofluorene. Characteristics of the Lesions in Albino Rats. Cancer Research, **7**:647–57, 1947.
6. CUNNINGHAM, B., and KIRK, P. L. Measure of Growth in Tissue Culture. J. Cell. & Comp. Physiol., **20**:343–58, 1942.
7. CUNNINGHAM, L.; GRIFFIN, A. C.; and LUCK, J. M. The Desoxyribonucleic Acid Content per Nucleus in Normal, Precancerous, and Cancerous Tissues of the Rat. Cancer Research, **10**:211, 1950.
8. DAVIDSON, J. N. Some Factors Influencing the Nucleic Acid Content of Cells and Tissues. Cold Spring Harbor Symposia Quant. Biol., **12**:50–59, 1947.
9. ———. Nucleoproteins, Nucleic Acids and Derived Substances. Ann. Rev. Biochem., **18**:155–90, 1949.
10. ———. The Biochemistry of the Nucleic Acids, pp. 1–163. London: Methuen & Co. Ltd.; New York: John Wiley & Sons, Inc., 1950.
11. DAVIDSON, J. N., and LESLIE, I. A New Approach in the Biochemistry of Growth and Development. Nature, **165**: 49–53, 1950.
12. DAVIDSON, J. N.; LESLIE, I.; SMELLIE, R. M. S.; and THOMSON, R. Y. Chemical Changes in the Developing Chick Embryo Related to the Deoxyribonucleic Acid Content of the Nucleus. Biochem. J., Vol. **46**, Proc. xl, 1950, and unpublished results.
13. DAVIDSON, J. N.; LESLIE, I.; and WAYMOUTH, C. The Nucleoprotein Content of Fibroblasts Growing *in Vitro*. 4. Changes in the Ribonucleic Acid Phosphorus (RNAP) and Deoxyribonucleic Acid Phosphorus (DNAP) Content. Biochem. J., **44**:5–17, 1949.
14. DAVIDSON, J. N.; LESLIE, I.; and WHITE, J. C. Studies on the Average Content of Nucleic Acids in Human Marrow Cells. Biochem. J., Vol. **47**, Proc. xvi, 1950, and unpublished results.
15. DAVIDSON, J. N., and MCINDOE, W. M. Phosphorus Compounds in the Cell Nucleus. Biochem. J., Vol. **45**, Proc. xvi, 1949, and unpublished results.
16. DAVIDSON, J. N., and RAYMOND, W. Nucleic Acids Labeled with P^{32} and N^{15}. Biochem. J., Vol. **42**, Proc. xiv, 1948.
17. DAVIDSON, J. N., and WAYMOUTH, C. Factors Influencing the Nucleoprotein Content of Fibroblasts *in Vitro*. Biochem. J., **37**:271–77, 1943.
18. ———. Nucleic Acids and Tissue Growth. Nutrition Abstr. & Rev., **14**:1–18, 1944.
19. ———. Nucleoprotein Content of Fibroblasts Growing *in*

Vitro. II. Effect of Tissue Extracts. Biochem. J., **39**:188–99, 1945.

20. Furst, S. S.; Roll, P. N.; and Brown, G. B. On the Renewal of the Purines of the Desoxypentose and Pentose Nucleic Acids. J. Biol. Chem., **183**:251–66, 1950.
21. Geschwind, I., and Li, C. H. The Nucleic Acid Content of Fetal Rat Liver. J. Biol. Chem., **180**:467–71, 1949.
22. Greenstein, J. P. Biochemistry of Cancer, p. 367. New York: Academic Press Inc., 1947.
23. Griffin, A. C.; Cook, H.; and Cunningham, L. Tissue Proteins and Carcinogenesis. III. Precancerous Change in the Liver and Serum Proteins of Rats Fed Acetylaminofluorene. Arch. Biochem., **24**:190–98, 1949.
24. Griffin, A. C.; Nye, W. N.; Noda, L.; and Luck, J. M. Tissue Proteins and Carcinogenesis. J. Biol. Chem., **176**: 1225–34, 1948.
25. Hammarsten, E., and Hevesy, G. Rate of Renewal of Ribo- and Desoxyribo-Nucleic Acids. Acta physiol. Scandinav., **11**:335–43, 1946.
26. Hevesy, G. Nucleic Acid Metabolism. Advances Biol. & M. Physics, **1**:409–54, 1948.
27. Hull, W., and Kirk, P. L. Tissue Culture Studies. II. The Relationship of Nucleic Acid Increase to the Growth of Cells *in Vitro*. J. Gen. Physiol., **33**:325–34, 1950.
28. Huxley, J. S. Constant Differential Growth Ratios and Their Significance. Nature, **114**:895–96, 1924.
29. Jacobj, W. Über das rhythmische Wachstum der Zellen durch Verdopplung ihres Volumens. Arch. Entwicklungsmech. Organ., **106**:124–92, 1925.
30. Klein, E.; Kurnick, N. B.; and Klein, G. The Effect of Storage in the Nucleic Acid Content and Virulence of Mouse Ascites Tumor. Exper. Cell Research, **1**:127–34, 1950.
31. Mandel, P.; Jacob, M.; and Mandel, L. Étude sur le nombre de noyaux hépatiques au cours de l'inanition protéique prolongée. Compt. rend. Acad. d. sc., **229**:1370–71, 1949.
32. ———. Étude sur la métabolisme des acides nucléiques. 1. Action du jeûne protéique prolongé sur les deux acides nucléiques du foie, du rein, et du cerveau. Bull. Soc. chim. biol., **32**:80–88, 1950.
33. Mark, D. D., and Ris, H. A Comparison of Desoxyribonucleic Acid Content in Certain Nuclei of Normal Liver and Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**: 727–29, 1949.
34. Mirsky, A. E., and Ris, H. Variable and Constant Components of Chromosomes. Nature, **163**:666–67, 1949.
35. Needham, J. Chemical Embryology. Cambridge: At the University Press, 1931.
36. ———. Heterogony and the Chemical Ground Plan of Animal Growth. Nature, **130**:845–46, 1932.
37. ———. Chemical Heterogony and the Ground Plan of Animal Growth. Biol. Rev., **9**:79–109, 1934.
38. Novikoff, A. B., and Potter, V. R. Changes in Nucleic Acid Concentration during the Development of the Chick Embryo. J. Biol. Chem., **173**:233–38, 1948.
39. Price, J. M.; Miller, E. C.; Miller, J. A.; and Weber, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. Cancer Research, **9**:398, 1949.
40. Price, J. M.; Miller, E. C.; Miller, J. A.; and Weber, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3′-Methyl-, 2′-methyl-, and 2-methyl-4-dimethylaminoazobenzene and 4′-fluoro-4-dimethylaminoazobenzene. Cancer Research **10**:18–27, 1950.
41. Ris, H., and Mirsky, A. E. Quantitative Cytochemical Determination of Desoxyribonucleic Acid with the Feulgen Nucleal Reaction. J. Gen. Physiol., **33**:125–46, 1949.
42. Schlenk, F. Chemistry and Enzymology of Nucleic Acids. Adv. Enzymol., **9**:455–535, 1949.
43. Schmidt, G.; Hecht, L.; and Thannhauser, L. J. The Behavior of the Nucleic Acids during the Early Development of the Sea Urchin Egg. J. Gen. Physiol., **31**:203–8, 1949.
44. Schrader, F., and Leuchtenberger, C. Variation in the Amount of Desoxyribose Nucleic Acid in Different Tissues of Tradescantia. Proc. Nat. Acad. Sc. **35**:464–68, 1949.
45. Signorotti, B.; Hull, W.; and Kirk, P. L. Tissue Culture Studies. I. Standardization of Technique for Quantitative Culturing. J. Gen. Physiol., **33**:315–25, 1950.
46. Teissier, G. Recherches morphologiques et physiologiques sur croissance des insectes. Tr. Stat. Biol. Roscoff., **9**:27–238, 1931.
47. Tompkins, E. R.; Cunningham, B.; and Kirk, P. L. Mitosis, Cell Size, and "Growth" in Culture of Chick Embryo Heart. J. Cell. & Comp. Physiol., **30**:1–14, 1947.
48. Vendrely, R., and Vendrely, C. La teneur du noyau cellulaire en acide désoxyribonucléique à travers les organes, les individus et les espèces animales. Experientia, **4**:434–36, 1948.
49. ———. Sur la teneur individuelle en acide désoxyribonucléique des gamètes d'oursins Arbacia et Paracentrotus. Compt. rend. Soc. de biol., **143**:1386–87, 1949.
50. ———. La teneur du noyau cellulaire en acide désoxyribonucléique à travers les organes, les individus et les espèces animales. Experientia, **5**:327–29, 1949.
51. ———. Sur la teneur absolue en acide désoxyribonucléique en organe cellulaire chez quelques espèces d'oiseaux et de poissons. Compt. rend. Acad. d. sc., **230**:670–71, 1950.
52. Willmer, E. N. Carbohydrates Metabolism of Chick Fibroblasts *in vitro*. J. Exper. Biol., **18**:237–56, 1942.

Isolation of Fungi from Transplanted, Chemically Induced and Spontaneous Tumors

I. General Considerations*

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The microscopic study of a variety of tumors, by means of cytological technics not ordinarily applied to such tissue (19), has led to the observation of spores and other fungal structures in all types of neoplasms examined. These structures were noted initially in the debris of tumors degenerating in response to chemotherapeutic agents; later, by means of special stains, they were studied in the intact tissue and found to be intracellular or even intranuclear. Attempts to isolate the organisms from tissue were successful in so many types of neoplasm, including human tumors and spontaneous tumors of the mouse, that we have become convinced that they must play some significant role—even if only a secondary one; and studies were undertaken in the hope of elucidating these relationships. The present paper deals with our general observations; the study of specific tumors will be reported later.

MATERIALS AND METHODS

Conidia and other plant cells were demonstrated in tissue by means of smears and paraffin sections subjected to a variety of cytological technics. Among these were: acetic orcein (18), Benda-Kull (48), Robinow (37), Protargol (32), Papanicolaou (33), Chlorazol Black E. (13), and also such well known technics as Flemming's tri-color following Flemming fixation, the Feulgen reaction, iron-hematoxylin (particularly in sections), and the Pianese technic for demonstration of fungus in tissue. Dry tissue imprints of blood-forming organs and of tumors were stained with Giemsa, May-Grünwald, and Robinow. Fixatives containing osmic acid were the most favorable for differentiating fungal elements, and Protargol was particularly good for the demonstration of delicate membranes and other characteristics of spores inside the host cells. The pre-hydrolysis employed in the Robinow technic produced extremely clear pictures of vegetative growths *in situ* (Figs. 1, 2) when used with smear preparations. Only dissociated fungal parts were revealed when the other technics were applied to smears. Sections were less useful than smears in these examinations except for the study of dissociated cells, since the patterns of the complex plant structures were difficult to reconstruct. This might explain why they have not been more commonly observed.

Tumors from animals previously immersed in an iodine-alcohol solution were dissected inside a Plexiglas transfer chamber. The tissues were streaked on Petri plates of Sabouraud's agar (4 per cent glucose, 3 per cent peptone, and 2 per cent bacto-agar, adjusted to pH 5.5). Uninoculated control plates exposed to the air of the transfer chamber were incubated with every series of tests as an aid in detecting the presence of possible air-borne contaminants (usually Penicillium and Aspergillus.) Syncephalastrum and other forms which were recovered regularly from tumor tissue have never been found on the control plates. Sabouraud's agar is not favorable for bacterial growth, and we but rarely found bacterial forms among tissue isolates and never in the controls. Littman's ox-gall agar (28) has also been used with considerable success in primary isolations, although the fungi produce aerial mycelium more slowly on Littman's than on Sabouraud's. Adjustment of pH is not required with this medium. Early growth is colonial, which facilitates separation if more than one organism appears on the plate.

Initially, the cultures were incubated at approximately the body temperature of mice (37° C.). Later, it was observed that fungi were more readily isolated from tissue when the incubation temperature was slightly lower (room temperature to 33° C.), although the cultures, once isolated, could be transferred to slants, where they grew abundantly at 37° C.

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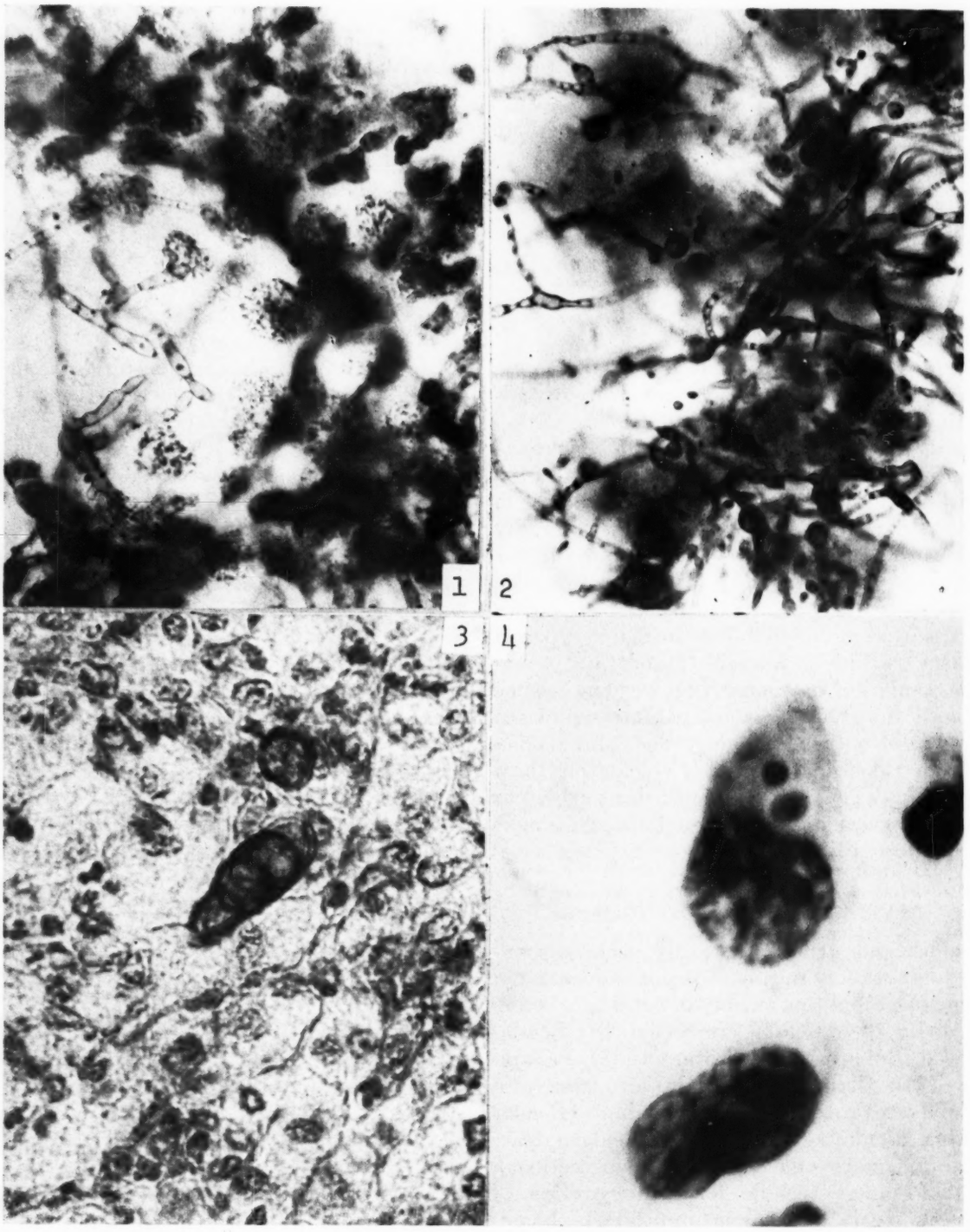


FIG. 1.—Robinow smear of Sarcoma 37, showing fungus mycelium interspersed between tumor cells. × 900.

FIG. 2.—Robinow smear of a methylcholanthrene-induced fibrosarcoma (C3H strain mice) similar to Fig. 1. × 900.

FIG. 3.—Paraffin section, stained with hemalum and eosin, of a growth induced in the leg muscle of a C3H mouse by injection of a suspension of Alternaria spores. × 900.

FIG. 4.—Robinow stained dry tissue imprint of Sarcoma 37 cell showing bodies that have penetrated into, or have been engulfed by, the cytoplasm. × 900.

The materials tested were transplantable mouse sarcomas 37 and 180, a transplantable carcinoma originating in our own Institute, transplantable leukemia, tumors induced in A and C3H strain mice by subcutaneous injections of methylcholanthrene, spontaneous mammary carcinomas from C3H mice, and spontaneous lymphatic leukemia from MacDowell's C-58 stock. Normal tissues (heart, kidney, lungs, intestinal wall, stomach, liver, ovary and testis, mammary tissue, lymph nodes, spleen, blood, and thymus) of the host mice were also tested for the presence of fungus, both before and after the animals became tumor-bearing. Human tumor tissue (mammary carcinoma, lymph nodes of Hodgkin's disease, fibro- and lymphosarcomas), taken in sterile Petri dishes directly from the operating room following biopsy or surgery, were also cultured. Normal tissues from human hosts were not tested. Care was taken to choose spontaneous lesions that had not been open to the surface, and, in the case of transplantable tumors, to use young (7 days or younger) non-necrotic implants.

Fungus cultures from the agar plates were studied in cotton blue preparations, and identifications were made by means of this technic as well as by direct observation in the phase and light microscopes. Samples of each tissue to be tested were also prepared for microscopic examination by more permanent methods.

RESULTS

Isolations

Table 1 shows the types of microörganisms isolated from the different tumors and the results of culturing normal tissue controls. Occasionally, common laboratory contaminants such as *Aspergillus niger* and species of Penicillium were obtained from fibrosarcomas induced by carcinogens. It seems entirely possible that these forms were contaminants acquired through the ulceration so common in early stages of induction of chemically produced tumors.

TABLE 1

ISOLATION OF FUNGI

			Tumor tissue		Normal tissues of host		
Tumor	Type of tumor	Host	Fungus isolated	No. of positives	Organ	Fungus isolated	No. of positives
S-37	Trans.	CFW	Syncephalastrum	138	Intestine	Aspergillus	5
			Imperfect				
S-180	Trans.	CFW	Syncephalastrum	14	Intestine	Aspergillus	4
			Imperfect			Yeasts	2
Mammary ca.	Spont.	C3H	Syncephalastrum	43	Intestine	Syncephalastrum	6
			Alternaria		Stomach	"	1
					Ovary	Alternaria	1
					Mammary tissue	"	3
					Thymus	Syncephalastrum	1
MC-l rhabdomyosarcoma	Chem.-induced	C3H	Alternaria	3			
MC-le fibrosarcoma	Chem.-induced	C3H	Alternaria	18			
MC-lb fibrosarcoma	Chem.-induced	C3H	Alternaria	1			
			Imperfect (Geotrichum)	1			
Fibrosarcoma	Chem.-induced	"A" strain	Imperfect (unidentified)	6	Intestine	Penicillium	1
Leukemia	Spont.	C-58	Yeastlike	22	Intestine	Penicillium	2
					Stomach	"	2
					Blood	Yeastlike	5
					Liver	"	4
					Thymus	"	1
					Spleen	"	11
Leukemia	Trans.	AKm	Yeastlike	37	Intestine	Aspergillus	2
					Blood	Yeastlike	continually
					Liver	"	"
					Spleen	"	"

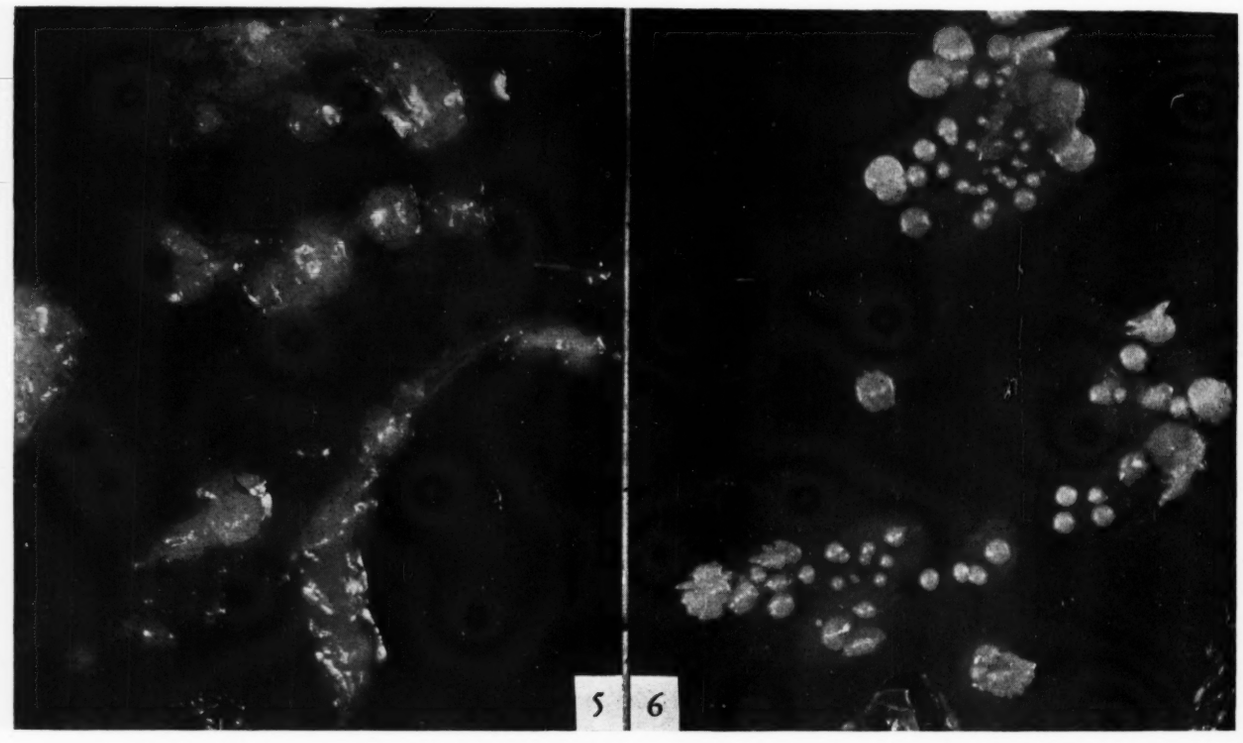
Normal tissue controls.—None of the normal body tissues, except the intestine of Swiss mice used for the implantation of Sarcoma 37 and Sarcoma 180, produced any fungi on Sabouraud's agar, either before or after the implantation of the tumors. The intestinal forms were usually species of Aspergillus and could be found in unimplanted as well as implanted mice, although never in the tumor. Syncephalastrum, always present in Sarcoma 37, was never recovered from intestinal or any other tissue of Carworth Farms mice. Apparently no infection of the normal tissue from the tumors occurred during the period of survival of the host after implantation.

Microscopic preparations made by the Benda-Kull method showed occasional spores lodged in the tissue of the intestinal wall of nursling C3H

mice, but no other tissues of these young mice showed spores, nor did they yield fungus of any kind when cultured. The intestine from 6-week-old mice was still the only source of fungus. After the first pregnancy, fungus could be cultured from mammary tissue, as well as from the intestine, even though the mouse was not yet tumor-bearing. The same fungus was once or twice derived from ovarian tissues in old age, after tumors had appeared; but at no time were we able to isolate fungus of any kind from the blood of C3H mice, and only once from the thymus. The remainder of the blood-forming organs and other body tissues yielded no fungus when streaked on Sabouraud's agar. Alternaria was found only in the tumor and in no mouse stock other than C3H.

Mouse tumors.—Two hundred and sixty-six Petri plate isolations of mouse tumors have been studied, with appropriate controls. All the spontaneous and chemically induced tumors and all but a few of the untreated transplanted tumors have yielded fungus. Mycelium is not always produced, but small glistening colonies usually can be detected microscopically in the tissues streaked on agar (Fig. 5), even in the absence of aerial filaments. The possibility that the organisms isolated on culture plates might be extrinsic contaminants is considerably decreased by such observations, since it is clear that the organisms are confined to the tumor tissue and do not appear on the surrounding medium.

In the case of mouse leukemias, all tissues (with the exception of the heart) tested from obviously moribund C-58 leukemic mice yielded a yeastlike growth that formed smooth, white, moist colonies (Fig. 6) on Sabouraud's agar and reluctantly produced aerial mycelium in cultures of lung, blood, and intestine. These grew so slowly that the agar became dry and cracked. When transferred to slants, they showed a tendency to revert to yeastlike forms. Microscopic examination of living unstained cultures showed that these are also very similar to the Fungi Imperfecti isolated from other mouse tumors. In most cases genera are still undetermined, but apparently they are all closely related. Similar yeastlike forms that produced a delicate nonsporulating mycelium have also been obtained from the lymph nodes, liver, spleen, and



FIGS. 5 and 6.—Primary Petri dish isolations showing that the fungus is growing up through the smeared tissue and not as peripheral contaminants.

FIG. 5.—Tissue of positively diagnosed Hodgkins' nodes streaked on Sabouraud's agar. Minute white patches mark areas where fungus is beginning to put up aerial filaments (2 weeks after tissue was plated).

FIG. 6.—Yeastlike fungus colonies growing on Littman's ox-gall agar from which the original streak of blood cells has disappeared completely (10 days at room temperature). This is a primary isolate from leukemic blood of C-58 mice.

kidney of AKm mice transplanted with leukemic spleen (courtesy of Dr. Burchenal of the Sloan-Kettering Institute).

Human tumors.—Our observations on human neoplasms are fragmentary and limited to random samples of specimens obtained from the biopsies of patients undergoing chemotherapy. This was further restricted by the necessity of using only tumors that were not obviously infected or superficially eroded. The most abundant materials available were mammary carcinoma, swollen nodes from positively diagnosed Hodgkin's patients, lymphosarcoma, and one fibrosarcoma. A suspected Hodgkin's node, tentatively diagnosed as a pyogenic granuloma, was also tested. The fungi isolated from human tumors were all of the group of Fungi Imperfecti (nonsporulating). Microscopic study of human mammary carcinomas and of several types of sarcoma suggests that they are allied to the Blastomycetes (Figs. 7–10).

From the granulomatous nodes and from several positively diagnosed Hodgkin's nodes, nonsporulating growths with abundant glistening white mycelium were obtained. The cultures turned a delicate pink after a few days' culture because of the production of numerous red droplets. Evidence of sporulation has been found in one or two of the specimens after numerous transfers, and these are now being grown on other media, in the hope of stimulating formation of identifiable spore structures.

All types of human neoplasms thus far tested have produced fungus growth when streaked on agar. Furthermore, spore forms are microscopically demonstrable in many types of human tumor that we have not yet tried to culture. Bacterially infected specimens, or specimens that had recently been subjected to some form of physical or chemical therapy, were usually negative. Preliminary indications are that, in biopsy material taken a few days after treatment with bacterial polysaccharide, fungal growth in culture is suppressed or much delayed. We are currently performing a series of experiments in mice, aimed at testing this further.

Pathogenicity of Organisms Obtained from Tumors

Spore suspensions of both Syncephalastrum and Alternaria, when freshly isolated from tumor tissue and injected either intraperitoneally or intramuscularly into our albino or C3H mice, were pathogenic. Animals thus injected died within 4 days, without exception. All normal tissues from these mice, cultured post mortem on Sabouraud's agar, yielded abundant colonies of fungus. There was a marked shrinkage of the spleen in each case. Virulence was reduced when the organisms were subcultured for several generations. Suspensions of spores from subcultures injected into mice produced firm lumps of tissue (granulomas?) but no deaths. Figure 3 shows partially disintegrated sporangia and spore cells (at tips of arrows) growing in the lesions thus produced.

Microscopic Observations

The tissues of tumors from which fungi were isolated were prepared for microscopic study by means of one or more of the technics mentioned above. In the control tumors, sectioned in paraffin and stained with hemalum and eosin, there were always found, scattered throughout the tissue, small round or oval cells that resembled small lymphocytes. These were never observed to divide mitotically, but in acetic orcein smears of Sarcoma 37 it was noted that some of them appeared to be subdividing within a membrane; and they were also recognized in 7-day implants stained with hemalum and counterstained with fast green. Moreover, the structure that had been mistaken for a nucleus surrounded by a very minute bit of cytoplasm was found to be an entire cell with a double-contoured or thickened wall. In addition, there were present smaller round bodies with deeply staining rims, each containing centrally located, endosome-like concentrations. These either became invasive or were phagocytized and were found in the cytosome (Fig. 4), or occasionally in the nucleus, of the tumor cells. In Benda-Kull preparations of smears of Sarcoma 37, delicate hyphal filaments were shown to run from one cell to another, sometimes penetrating into the cell and attaching to the nucleus, which then became distorted and at times was withdrawn completely from the cell body. Such enucleated cells are found frequently in areas of the tumor usually described as degenerating by "spontaneous necrosis."

DISCUSSION

No references to the demonstration of mycelium in tissue cultures, or even to any structures which were recognized as fungal, have come to our attention, although Grand (23) grew Hodgkin's lymph nodes in tissue culture, and by means of vital staining demonstrated the presence of specific cytoplasmic inclusions which were not found in the cells of corresponding normal tissues grown *in vitro*. Cell-free supernatant fluid from the Hodgkin's node cultures, added to cultures of normal lymph nodes, caused the appearance, in the cytoplasm of normal cells, of inclusions similar to those found in the original cultures. In personal talks with those who study tissue cultures of

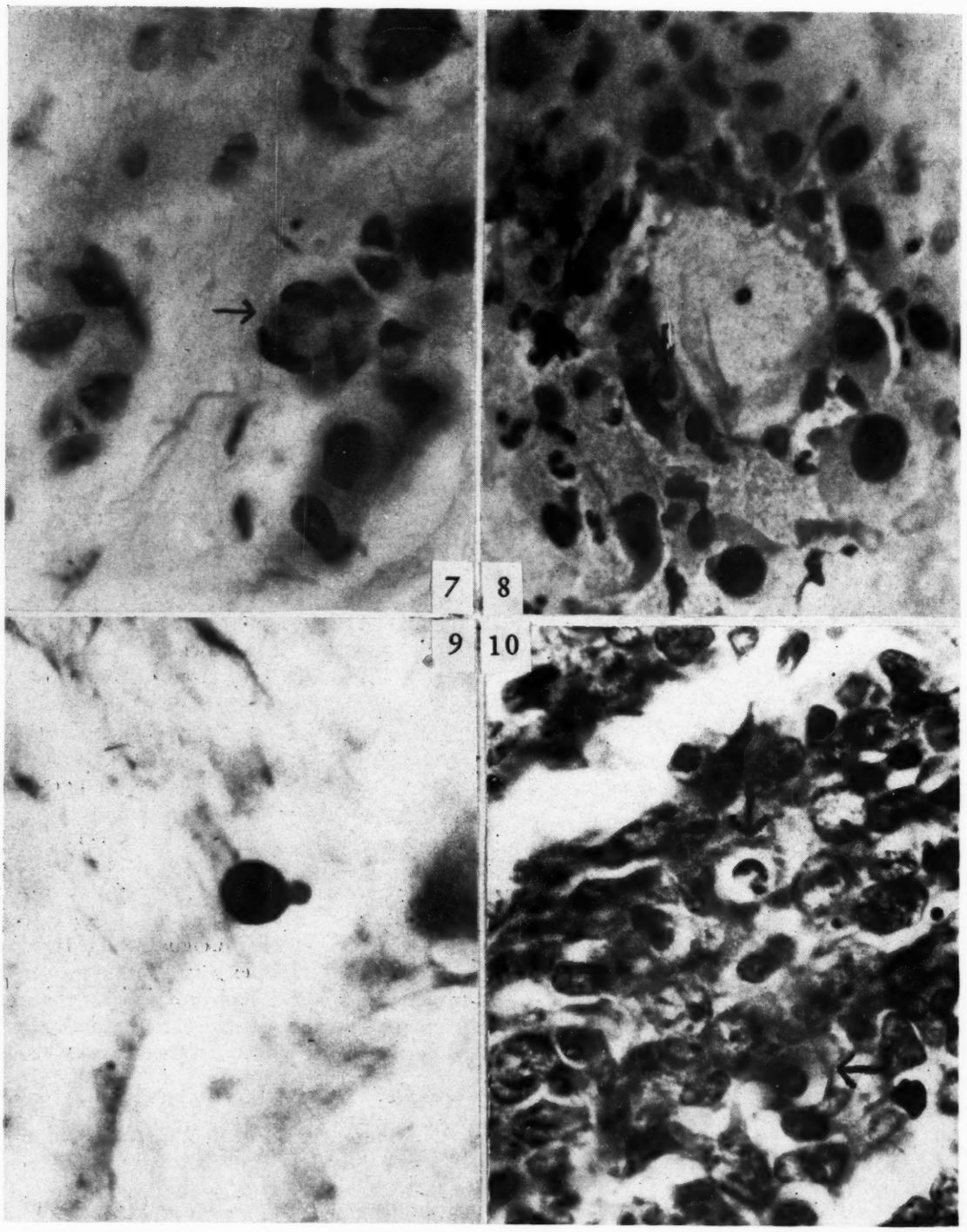


FIG. 7.—Paraffin section, stained in hemalum and eosin, of a human uterine carcinoma. Parasitized nucleus (organism unidentified) at tip of arrow. × 900.

FIG. 8.—Papanicolaou smear of a primary carcinoma of the human cervix uteri. Note parasitized cell at tip of arrow. × 900.

FIG. 9.—Paraffin section of a spontaneous mammary tumor from an "A" strain mouse showing budding spore, greatly magnified, as demonstrated by the McManus stain for carbohydrates, in which background tumor cells remain completely unstained. × 1,800.

FIG. 10.—Paraffin section of a human fibrosarcoma. Yeastlike fungus spores, enclosed in capsules, at tips of arrows. × 900.

neoplastic cells I have learned that it is standard practice to treat cultures of tumor tissue with antibiotics and to grow them at high temperatures in order to arrest the growth of fungal "contaminants." The media employed are, furthermore, quite different from those needed to further isolation of fungus from tissues. Tissue cultures are ordinarily grown at high pH, whereas isolation of most fungi is accomplished on an acid medium. Voegtlin (54) has shown that tumor tissue is definitely more acid than the surrounding normal tissues. In hepatomas the pH dropped to 6.4 in rats (6.2 in mice), when starved host animals were supplied with excess glucose, while the pH of the liver (7.4) remained unchanged. Essentially the same findings were made earlier by Cori and Cori for blood passing through tumors.

The presence of bodies in cancer cells which did not appear to be normal cytoplasmic constituents was noted by many investigators in the nineteenth century (1, 2, 4, 11, 20, 34, 36, 43, 49, 50, 51, 52) and, more recently, by Burns and Schenken (7). Some of these authors considered that the inclusions were of fungal origin, while many others who observed similar inclusions (references not given) thought they were protozoa.

A number of authors, believing the intracellular bodies to be fungi, were successful in culturing the organisms outside the body (5, 6, 8, 14, 15, 29, 34, 35, 38, 39, 40, 41, 42, 44, 45, and 46 as well as many others listed in the *Index Medicus* from 1895 to 1900). An excellent review of the literature on the relation of yeasts to malignant tumors is that of Defendorf (17). Within the past few years, several papers on the association of Blastomycetes with lymphosarcoma and Hodgkin's nodes have appeared (10, 12, and 16). Contemporary authors who have isolated organisms other than Blastomycetes are Leyton (27), Mori (31), and Gerlach (21). The latter author has obtained, from hundreds of human and animal tumors, microscopic fungi which he calls "*Micromyces blastogenes*," following the terminology of Mori. Both Mori and Gerlach believe that these fungi have a filterable phase during which they behave like viruses, a hypothesis similar to that of Glover (22). Wuerthele-Caspé (56) is currently isolating from all types of human and animal tumors organisms which appear to be similar to those described by Gerlach. However, since they are acid-fast in some phase of their life cycle, she believes they are Mycobacteria.

These are but a few examples, of the many that might be chosen from the literature, showing how commonly inclusions—which some investigators regard as structures intrinsic to the malignant cell, others as metabolic products, and still others as microörganisms—have been observed in cancer tissue. Whatever their significance, their presence must be reckoned with, not only in terms of the cytological picture, but also of the chemical relationships or differences existing between the tumor tissue, in which these organisms are carrying out their life processes, and the normal tissues of the tumor host. Preliminary investigations made by us on the mode of action of chemotherapeutic agents have shown that the fungi respond to some of them, adding their debris to that of degenerating host cells and complicating the evaluation of experimental results.

What influence do these organisms have on tumor growth? Microscopic observations indicate that the destructive action of these parasites may account for much of the "spontaneous necrosis" observed in transplanted tumors. Are they merely contaminants that find a favorable environment (e.g., pH) in tumor tissue? If so, what are the properties of tumor tissue that render it particularly susceptible to invasion by this specific type of organism? Wharton (55) reported that, after injection of various antigens, tumor-bearing mice have a lower antibody titer than nontumor-bearing mice. Has this any bearing on the problem? Antigenic studies have been begun in collaboration with him to investigate some of these possible relationships.

At the present stage of our study of these phenomena we are not prepared to attempt any explanations of the presence or role of fungi in tumor tissue. The evidence of past investigations and of our own observations, however, point to their prevalence in many, if not all, types of tumor; and an extensive survey of the literature has revealed no conclusive evidence that microörganisms may not be causative agents. The large number of earlier papers were referred to by Haddow (25) as "a depressing catalogue of allegedly specific pathogens." "Nevertheless," he continues, "the logical possibility exists and must therefore very willingly be admitted, that the biology of cancer may in some way be bound up with processes of infection." The recent reports of Mann and Dunn (30) in England, that they have been able to transmit several mouse tumors from completely desiccated tissue following exposure to low temperatures (−79° C.), focus attention still further on the possibility ol what these investigators call "a continuing cause."

SUMMARY

The microscopic study of smear preparations of tumor tissue, prepared by a variety of cytological

technics, has revealed the presence of inter- and intracellular fungal structures. Spores and developing conidia may be easily mistaken, in preparations made by routine histological technics, for lymphocytes and other blood cells.

Mycelial forms of the fungi were readily isolated from transplanted, induced, and spontaneous tumors of the mouse and from human tumors. Fungi Imperfecti related to the Blastomycete group have been isolated from all types of tumor, including human neoplasms, and from mouse leukemias. In mice, a second form of fungus, apparently specific for the host strain, is frequently encountered.

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REFERENCES

1. Aievoli, Eriberto. Richerche sui blastomiceti nei neoplasmi. Centralbl. f. Bakt., **20**:745–49,1896.
2. Alexeieff, A. Sur un chlamydozoaire parasite des protozoaires, sur le chlamydozoaire du cancer. Arch. de zool. expér. et gén., Notes et Rev., Par., 1912, X, pp. ci–cix.
3. Becker, S. W.; Kahn, D.; and Rothman, S. Cutaneous Manifestations of Internal Malignant Tumors. Arch. Dermat. & Syph., **45**:1069, 1942.
4. Bosc, F. J. Les parasites du cancer et du sarcome. Compt. rend. Acad. d. sc., **126**:541–44, 1898.
5. Bra, M. D'un champignon parasite du cancer. Compt. rend. Soc. de biol., **5**:1050–53, 1898.
6. Braithwaite, J. On the Micro-organism of Cancer. Lancet, **1**:1636–38, 1895.
7. Burns, E. L., and Schenken, J. R. Spontaneous and Primary Hepatomas in Mice of Strain C3H. IV. A Study of Intracytoplasmic Inclusion Bodies and Mitochondria. Cancer Research, **3**:697, 1943.
8. Busse, O. Ueber Saccharomycosis hominis. Arch. f. path. Anat., **140**:23–46, 1895.
9. ———. Experimentelle Untersuchungen ueber Saccaromycosis. *Ibid.*, **144**:360–72, 1896.
10. Cawley, E. P., and Curtis, A. C. Histoplasmosis and Lymphoblastoma. Are These Diseases Related? J. Invest. Dermat., **11**:443, 1948.
11. Clarke, J. J. Protists and Disease. London: Baillière, Tyndall & Cox, 1922.
12. Cohen, M. Binocular Papilledema in Case of Torulosis Associated with Hodgkin's Disease. Arch. Ophth., **32**:477, 1944.
13. Conn, J. Chlorazol Black E. Stain Technol., **8**:189–92, 1943.
14. Curtis, F. Contribution à l'étude de la saccharomycose humaine. Ann. Inst. Pasteur, **10**:449–68, 1896.
15. ———. Note sur un nouveau parasite humain, *Megalococcus myxiodes*, trouvé dans un néoplasme de la région inguino-crurale. Compt. rend. Soc. de biol., **2**:715–18, 1895.
16. Da Silva L. C., *et al.* Blastomicose Sul-Americana associada a molestia de Hodgkin. Cancer Current Lit., **2**:574, 1948.
17. Defendorf, A. R. Yeasts and Their Relation to Malignant Tumors. Tr. Am. Micro. Soc., **18**:219–47, 1897.
18. Diller, I. C. Smear Methods for Mammalian Tissues Including Tumors. J. Tech. Methods, **25**:73, 1945.
19. ———. Fungi Associated with Tumor Tissues. Anat. Rec., **105**:24, 1949.
20. Foa, P. Ueber die Krebsparasiten. Centralbl. f. Bakt., **12**:185–92, 1892.
21. Gerlach, F. Krebs und obligater Pilzparasitismus. Wien: Urban & Schwarzenberg, 1948.
22. Glover, T. J. Bacteriology of Cancer. Canada Lancet & Practitioner, **67**:5, 1926.
23. Grand, C. C. Cytoplasmic Inclusions Characteristic of Hodgkin's Diseased Lymph Nodes in Tissue Culture. Cancer Research, **9**:183–92, 1949.
24. Hache, E. Les coccidies dans les cancers epithéliaux. Compt. rend. Soc. de biol., **42**:637–39, 1890.
25. Haddow, A. The Mode of Action of Chemical Carcinogens and Its Possible Relation to the Origin of Viruses. Growth, **11**:339, 1947.
26. Kolmer, J. A.; Harkins, M. A.; and Soleeby, E. An Ineffectual Attempt To Cultivate the Glover Organism from Carcinomas. J.A.M.A., **89**:1868, 1937.
27. Leyton, H. G., and Leyton, G. B. An Account of Some of the More Recent Scientific Investigations Which Gave Rise to the Treatment of Cancer with Allyl Isothiocyanate. M. Times, London, April, 1939.
28. Littman, M. L. A Special Microslide for Culture of Fungi. Am. J. Clin. Path., **19**:278–80, 1949.
29. Maffucci, A., and Sirleo, L. Ueber die Blastomyceten als Infectionserreger bei bösartigen Tumoren. Ztschr. f. Hyg., **27**:1–30, 1898.
30. Mann, I., and Dunn, W. J. Propagation of Mouse Carcinoma by Dried Tumor Tissue. Brit. M. J., **2**:255–57, 1949.
31. Mori, N. Filtrierbare Vira und Krebs im Einklang mit meiner Hypothese über deren myzeliale Natur. Der Krebsartzt. Wien: Urban & Schwarzenberg, 1949.
32. Moskowitz, N. The Use of Protein Silver for Staining Protozoa. Stain Technol., **25**:17–20, 1950.
33. Papanicolaou, G. N. Cytological Diagnosis of Uterine Cancer by Examination of Vaginal and Uterine Secretions. Am. J. Clin. Path., **19**:301–8, 1949.
34. Pfeiffer, L. Der Parasitismus des Epithelialcarcinoms. Centralbl., f. Bakt., **14**:118–30, 1893.
35. Plimmer, H. G. The Aetiology and Histology of Cancer. Practitioner, **62**:430–55, 1899.
36. Podowssoski, W., and Sawatschenko, J. Ueber Parasitismus bei Carcinomen nebst Beschreibung einiger in den Carcinomgeschwülsten schmarotzenden Sporozoen. Centralbl. f. Bakt., **11**:493–500, 1896.
37. Robinow, C. F. A Study of the Nuclear Apparatus of Bacteria. Proc. Roy. Soc., London, s.B, **130**:299–324, 1942.
38. Roncali, D. B. Sur l'existence des levures organisées dans les sarcomes. Ann. de microg., **8**:449–61, 1896.
39. ———. Klinische Beobachtungen und histologische und mikrobiotische Untersuchungen über einen Fall von primärem Adenocarcinom. Centralbl. f. Bakt., **24**:158–65, 1898.
40. ———. Di un nuovo blastomicete isolato da un epitelioma della lingua e dalle metastasi ascellari di un sarcoma della ghiandola mammaria. Centralbl. f. Bakt., **20**:481–88, 1896.

41. ———. Intorno all'esistenza di fermenti organizzati nei sarcomi. *Ibid.*, pp. 726–44, 1896.

42. ———. On the Existence of Blastomycetes in Adenocarcinomata and Sarcomata. J. Path. & Bact., **5**:1–13, 1898.

43. RUSSELL, W. A Characteristic Organism of Cancer. Brit. M. J., **2**:1356, 1890.

44. SANFELICE, F. Ueber die pathogene Wirkung der Sprosspilze. Centralbl. f. Bakt., **17**:625–34, 1895.

45. ———. Ueber die pathogene Wirkung der Blastomyceten. Ztschr. f. Hyg. u. Infektionskr., **29**:463–504, 1898.

46. ———. Ein weiterer Beitrag zur Aetiologie der bösartigen Geschwülste. Centralbl. f. Bakt., **24**:155–58, 1898.

47. SAWATSCHENKO, J., and PODOWSSOSKI, W. Weitere Untersuchungen über schmarotzende Sporozoen in den Krebsgeschwülsten. Centralbl. f. Bakt., **12**:17–28, 1892.

48. SCHRADER, F. The Kinetochore or Spindle Fibre Locus in Amphiuma. Biol. Bull., **70**:484–98, 1936.

49. SOUDEKEWITCH, J. Recherches sur le parasitisme intracellulaire et intranucléaire chez l'homme. Ann. Inst. Pasteur, **6**:145–57, 1892.

50. ———. Parasitisme intracellulaire des néoplasies cancéreuses. *Ibid.*, pp. 545–57, 1892.

51. THOMA, R. Ueber eigenartige parasitäre Organismen in den Epithelzellen der Carcinome. Fortschr. d. Med. Berl., **7**:413, 1889.

52. VIRCHOW, R. Die endogene Zellenbildung beim Krebs. Virchows Arch. f. path. Anat., **1–2**:197–225, 1855.

53. ———. Bemerkungen über die Carzinom-Zellen-Einschlüsse. *Ibid.*, **127**:188, 1892.

54. VOEGTLIN, C.; FITCH, R. H.; KAHLER, H.; JOHNSON, J. M.; and THOMPSON, J. W. The Influence of the Parenteral Administration of Certain Sugars on the pH of Malignant Tumors. J. Nat. Health Bull., **164**:1–14, 1935.

55. WHARTON, D. R.A. The Effects of Tumors on Antibody Levels in Mice. Cancer Research, **9**:628, 1949.

56. WUERTHELE-CASPÉ, V. Mycobacterial Forms in Tumor Tissue. N.Y. Micr. Soc. Bull., **2**:19–26; 215–18, 1948.

Inhibition of Plant Growth by Irradiation

V. Radiation Effects on Initiation and Completion of Growth*†

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INTRODUCTION

The main activity of a plant seedling is growth. During early development, growth is mainly longitudinal, so that a simple measurement of length reveals much about the growth processes. For this reason, plant seedlings have been widely used to study the effects of radiation on growth.

We have been studying the responses to radiation of Mung beans (*Phaseolus aureus*). We had previously investigated reactions of dry seeds and growing plants, studied dose-response relations, and attempted (unsuccessfully) to correlate disturbances of growth with changes in mitotic activity. The picture which is unfolding is far from simple: responses to radiation can be analyzed into a number of component reactions, which are altered differentially by changes in experimental conditions.

This paper deals with the growth of the first two parts of the plant which grow upward, i.e., the hypocotyl and the first segment of the epicotyl. We have observed the linear growth of the whole plant and of separate segments, after total or partial irradiation. Two components of the growth process, *growth initiation* and *growth completion*, are distinguishable in normal plants (section I). Effects of resecting various parts show that growth initiation depends on food reserves stored in the cotyledons, whereas growth completion is locally autarkic, i.e., independent of food supplied from other parts of the plant (section II). Growth is temporarily inhibited by moderate doses of x-rays (section III). In addition, there is a permanent effect, which reduces the total amount of growth after irradiation (section IV). The stunting effect is manifested in reduced capacities both of completing (section V) and of initiating growth (section VI). Effects of partial irradiation show that growth completion (section IV), as well as growth initiation (section VII), is affected only by direct irradiation. The "Discussion" deals with the nature of the three radiation reactions studied.

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EXPERIMENTAL AND RESULTS

Materials and methods have been described in detail in the first paper of this series (3). Mung beans (*Phaseolus aureus*) of a pure strain were used. The plants were grown in an ordinary room, with the roots in test tubes filled with tap water. Irradiation was administered with 100 kilovolt peak, with no filter; the dosage rate was usually 145 r/min in most of the tests described in section VI. The tests in sections V and VII were done with 150 kilovolt peak, with no filter, and with dosage rates of about 800 r/min.

I. Normal Growth: Phases of Initiation and Completion

This investigation deals with the linear growth of the first two segments which grow upward: the hypocotyl, which extends from the crown to the cotyledons; and the epicotyl (more accurately, the first epicotylar internode) between cotyledons and primary leaves (Fig. 1). Each of these two segments has a period of fast growth. The rate of combined upward growth remains unchanged as the growth wave passes from the hypocotyl across the insertion of the cotyledons into the epicotyl (Fig. 2).

For a more detailed study, 1-mm. segments were marked off with India ink (Fig. 1). The painting itself has no effect on growth. In individual segments, we studied *instantaneous growth rates* and *total growth capacity*. The former is defined as per cent elongation per day; the latter as the per cent

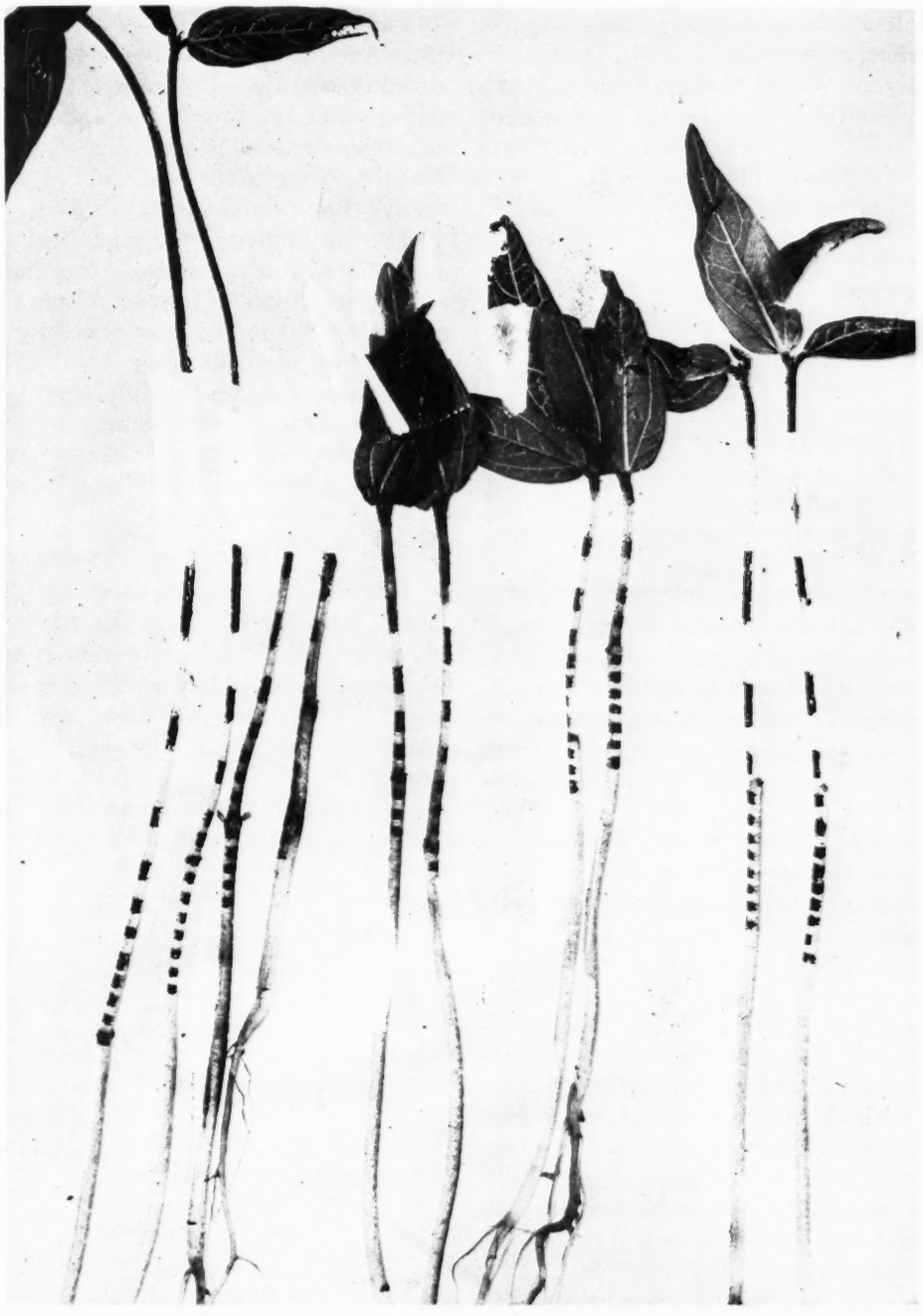

FIG. 1.—Segmental growth. Segments of 1-mm. width were marked at the time of treatment by painting alternate strips with India ink. Photographs were taken after growth had ceased. Plants are lined up at the beginning of the 3d segment, 2 mm. from the top at the time of marking. (*a*) Controls: note that most of the growth has occurred in the top 2 segments. (*b*) Resection of top 2 mm.: wound effect reduced growth in 2 segments adjacent to the wound. Normal growth completion below. Cotyledons transformed into vestigial leaves. (*c*) Partial irradiation of top 2 mm. with 40,000 r: irradiated portion and neighboring 2 segments stunted, normal growth completion below. (*d*) 40,000 r to whole stem: top 2 segments as in *c* plus proportional stunting of all lower segments. (*e*) 4,000 r to whole stem: the top 2 segments are stunted (compare with *a*), but all lower segments grew normally.

elongation achieved from the time of marking to the final cessation of growth.

Growth rates are low at the tip, reach a maximum about 2–6 mm. below the tip, and decline farther down. Individual measurements vary considerably, but the general character of the growth rate gradient is about the same in every case. It

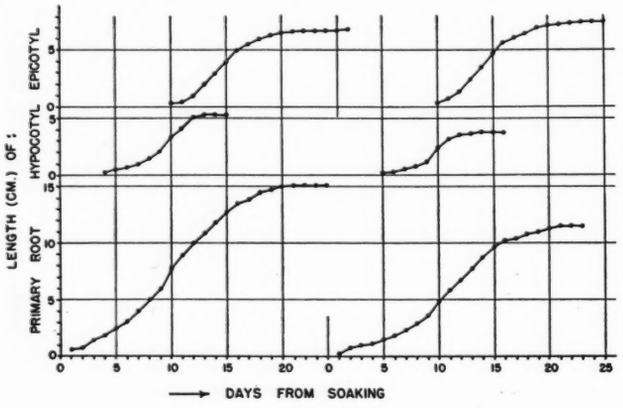

FIG. 2.—Normal linear growth

does not change as the growth wave passes from hypocotyl to epicotyl. Figure 3 shows mean growth rates observed in twelve beans on the day after marking.

The total growth capacity shows a different distribution. Most of the total growth capacity is located in the top 2 mm. (Fig. 1, *a*). Except for some 26 mm. contributed by the lower segments, the total growth capacity of the first 2 mm. is simply the difference between normal final length and length at the time of observation. The lower portions grow by limited amounts which are proportional to the growth rates and, therefore, decline exponentially with the distance from the tip. Figure 4 shows growth capacities of 25 beans measured in five experiments. The general trend and the individual variability are well demonstrated (the large spread in the top 2 mm. is due to indeterminacy and not to variability).

The tip contains most of the growth capacity, but does little actual growing; the lower segments show active actual growth, but their growth capacity is limited. Growth is *initiated* in the tip and *completed* in the lower segments. The capacity to initiate growth depends on the age (for a given set of experimental conditions); the capacity to complete growth depends on the position (during the phase of active growth) and can be described reasonably well by the equation:

$$G(s) = 51.7 \times e^{-0.344s} \times \Delta s \qquad (s > 2 \text{ mm.})$$

where $G(s)$ is the growth capacity of a segment of length Δs, at a distance s from the tip.

This equation yields a value of 26 mm. for the total amount of growth ready for completion located below the top 2 mm. We estimate the contribution of the top 2 mm. under the assumption that the capacity to complete growth is proportional to the growth rate, as it is in the lower segments. The resulting total is about 36 mm.

The variability of the capacity for growth completion was studied by analyzing the variability of total growth below the top 2 mm. The standard deviation within each experiment is about ±7 mm., or 30 per cent of the total growth. In addition, there are greater differences among experiments than can be explained by sampling variance; inter-experimental scatter accounts for an additional standard deviation between means of ±6 mm.

II. Effects of Resections

If the cotyledons are carefully amputated, the stem continues to grow about 40 mm. (mean of 16 observations). This is approximately the amount of growth ready for completion (as estimated above). Also, the growth mostly takes place at some distance from the tip. It appears that growth completion is autarkic, while growth initiation depends on the presence of the cotyledons (in beans of the age range used in our experiments).

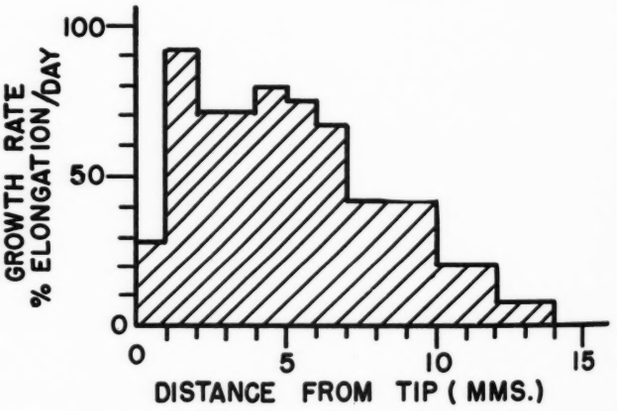

FIG. 3.—Growth rates expressed as additional growth/day/mm. Average of 12 beans.

If the top 2 mm. are cut off, the growth of the remaining segments is about normal, except for a small region below the wound (Fig. 1, *b*). Cutting off 4 and 6 mm. gives similar results, but the growth inhibition below the wound becomes more marked. With the exception of the region near the wound, the results agree with the hypothesis of local autarky of growth completion.

If the growing stem is cut up into 2-mm. segments, no significant further growth occurs. This

does not disprove the hypothesis of local autarky, because of the trauma involved, which, as pointed out above, inhibits growth in the region around the wound.

Growth initiation depends chiefly, but not exclusively, on the cotyledons. If the primary leaves

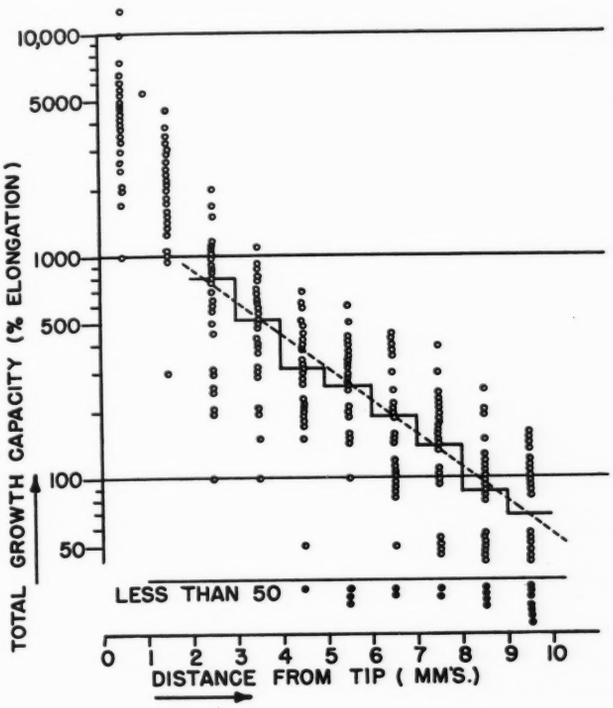

FIG. 4.—Segmental growth capacity. Final additional growth of 1-mm. segments. Each symbol represents one observation. Broken line, means. Dashed line, trend.

are resected, the result is a stunting of the epicotyl by about 9 mm. (mean of 25 observations). The hypocotyl provides some material for growth initiation if the cotyledons are cut at an early age; it is also involved in initiating growth in the primary root.

III. Temporary Inhibition of Growth Completion

After administration of moderate or high doses of x-rays, all visible growth stops for some time. After this interruption, growth proceeds continuously until the final length is reached (Fig. 5), without the intermediate stops characteristic of the radiation response of roots. Since the instantaneous growth rate depends on growth completion and not on growth initiation, the temporary inhibition must involve growth completion. Accordingly, it affects all growing segments. The larger the dose, the longer the duration of the inhibition is. With very large doses, the instantaneous inhibition of growth becomes irreversible. For instance, out of four plants irradiated with 110,000 r, three stopped growing permanently, and the fourth grew a few millimeters after an interruption of 10 days.

IV. Permanent Stunting: Estimation of Effects

After recovery from the temporary inhibition, an irradiated plant resumes its growth. However, the final length is less than normal: irradiation produces an irreversible reduction of the growth capacity. How this effect is measured depends on the way in which growth capacity is expressed. In dealing with effects of irradiation administered before any elongation has occurred, we found it convenient to express growth capacity in terms of final length. In the present investigation, the situation is more complicated. Should one consider the total length, or the additional growth after irradiation? Should hypocotyl and epicotyl be treated separately or combined? We have looked for an empirical answer in a number of tests in which we compared responses to graded doses of x-rays over a wide range of initial lengths. It was found that the one function of the dose which is largely independent of age is the *total elongation after irradiation* (of hypo- and epicotyl combined).

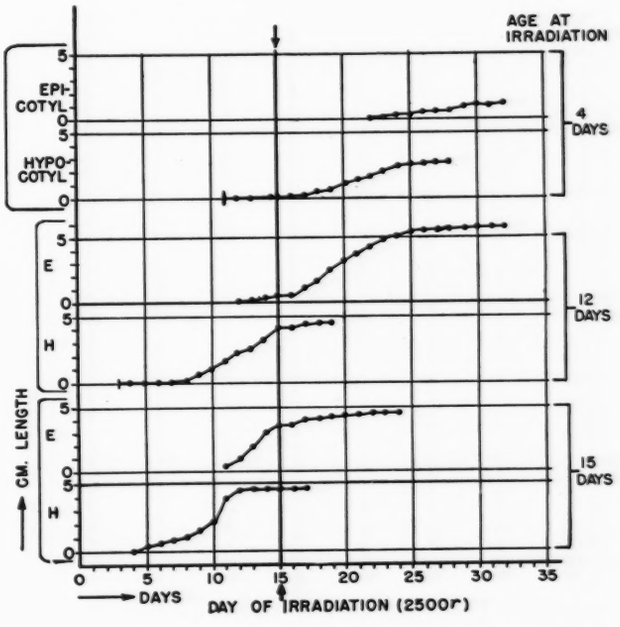

FIG. 5.—Linear growth after irradiation. Temporary inhibition and final stunting. For unirradiated plants, see Fig. 2.

V. Capacity of Growth Completion

Over a dosage range of from 0 to 4,000 r, the growth capacity is reduced from the normal value (which is about 180 mm. minus the actual length) to about 40 mm. The gradual abolition of these last 40 mm. occurs much more slowly over a dosage range reaching to nearly 100,000 r. The total

growth capacity thus consists of two portions of vastly different radio-resistance. We will discuss the more resistant portion first.

If a seedling is irradiated with a high dose (10^4 r), and segmental growth is studied, one finds that the reduction of growth capacity affects all segments (Fig. 1, *d*). This implies a reduction of the capacity to complete growth (this being the only growth capacity present in the lower segments).

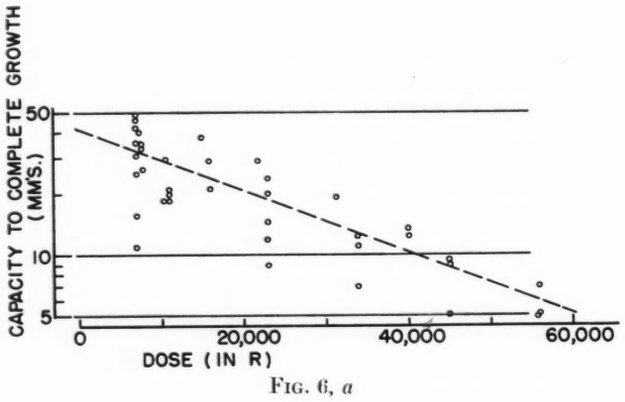

FIG. 6, *a*

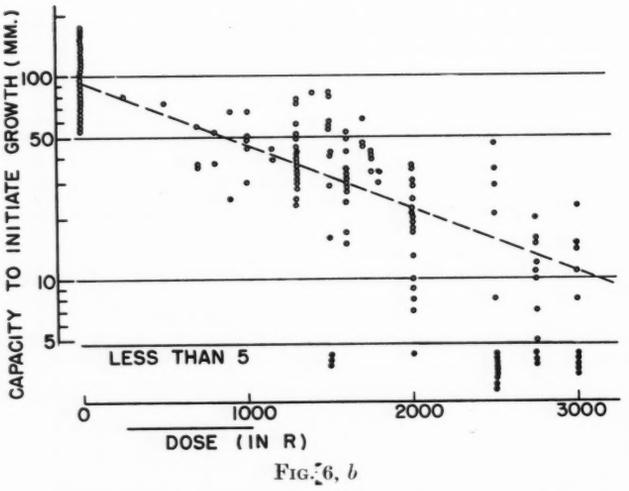

FIG. 6, *b*

FIG. 6.—Growth capacity after irradiation: (*a*) capacity to complete growth; (*b*) capacity to initiate growth, obtained by subtracting from the total growth capacity the mean capacity to complete growth for the dose given.

Each symbol represents the mean for a set of plants x-rayed with a given dose at a given time. Broken lines, trend.

The growth capacity is reduced proportionally in all segments; for instance, after irradiation with 11,000 r, each segment grows about 55 per cent of normal, except for the first 2 mm., where the abolition of growth initiation makes the stunting effect more pronounced. These findings suggest that we are dealing with a local effect. This was substantiated in experiments where only the top 2 mm. were x-rayed, the rest of the plant being carefully shielded against direct and, as far as possible, scattered radiation. The result is some stunting in the neighborhood of the irradiated segment, with normal growth occurring in the segments farther down (Fig. 1, *c*). The stunting in the immediate neighborhood of the irradiated segment is closely comparable to that obtained by cutting off the top 2 mm., and is probably a toxic effect rather than one due to scattered radiation.

The extent to which growth completion is reduced can be estimated by comparing total elongation in normal and irradiated plants, omitting the contribution of the portion which formed the top 2 mm. at the time of irradiation. One thus eliminates effects of growth initiation. One can also examine the effects of high doses only, obtaining growth completion in normal plants by extrapolation. Both methods lead to the same result: the capacity to complete growth declines exponentially with dose. Data concerning total growth after high doses are entered in Figure 6, *a*. It can be seen that a straight line in a semi-logarithmic plot fits the experimental points reasonably well, and that the estimate of the capacity to complete growth in normal plants, obtained by extrapolation to zero dose, agrees closely with the value arrived at on the basis of growth rates (section I) and of growth after resection of the cotyledons (section II).

VI. Capacity of Growth Initiation

The total growth capacity of a plant seedling has been divided into two components, namely, the capacities to initiate and to complete growth. The amount of the second, after irradiation, has been determined in the preceding section. The amount of the first is estimated by subtracting from the total additional growth after irradiation the growth due to completion, as estimated for the dose delivered (according to Fig. 6, *a*). In this manner, the data shown in Fig. 6, *b* were obtained.

The capacity to initiate growth is subject to considerable variations within and between experiments. The entries in Figure 6, *b* are group means. It can be seen that they vary considerably but also that they show a simple trend: the capacity to initiate growth declines exponentially with the increasing dose (and does so much faster than the capacity to complete growth).

Figure 6, *b* represents data from 1,712 irradiated beans, collected in 71 tests performed between 1946 and 1950. The dosage rates ranged from 145 to 1,400 r/min, and the peak potential from 100 to 150 kv. Beans with stem lengths, at irradiation, from 0.5 cm. to a few cm. below the final length are included. It is trivial that the relation does not hold for beans which have nearly reached their normal length; it is remarkable, however, that it does not hold, either, at the other end of the age

range—for beans in which the growth of the stem has not yet begun. In dry seeds and in young seedlings, a very different curve, with a definite threshold effect (3), can be noted. This implies that young seedlings have a mechanism for compensating radiation damage to the stem to a certain degree, a mechanism which is lost as soon as growth actually begins.

VII. Partial Irradiation

An experiment in which only a portion of the plant was x-rayed was described in the discussion of radiation effects on the capacity to complete growth. In this section, we are dealing with radiation effects on the capacity to initiate growth. This capacity is located in the tissues near the growing tip. It is lost if the top 2 mm., or the cotyledons, are removed. Thus, there are at least two regions involved in the initiation of growth. It is desirable, therefore, to investigate which region of the seedling must be irradiated in order to stop the initiation of growth.[1] A number of experiments were performed in which various regions were irradiated, both singly and in combinations, the remainder of the plant being carefully shielded. The dose was 4,000 r, which, if applied to the whole plant, abolished the initiation of growth but had little effect on the completion of growth.

One typical experiment with partial irradiation is summarized in Figure 7. The plants were divided into 3 segments: epicotyl, plus cotyledons, plus leaves; upper hypocotyl; and lower hypocotyl. These segments were x-rayed singly and in all combinations. Irradiation of the top portion was found to be necessary and sufficient to cause decided stunting.

In similar tests, we compared the effects of irradiation on the following regions: root, hypocotyl, epicotyl; alternating strips of 3-mm. width; epicotyl, cotyledons, and piece of stem on which cotyledons insert; leaves; and epicotyl. In all cases, marked stunting resulted if, and only if, the growing tip was directly irradiated.

DISCUSSION

Initiation and completion of growth.—The growth rate of a seedling is, essentially, the rate of net water increase. If a dry seed is soaked in water, a passive streaming-in of water occurs, and even a million roentgens do not slow down the swelling of the seed. On the other hand, a few hundred roentgens are sufficient to stop, temporarily, the growth of a seedling. In this case, the uptake of water is an active phenomenon, mediated through and roughly proportional to the amount of active protoplasm present. Radiation effects on longitudinal growth are based on changes in the amount of protoplasm produced.

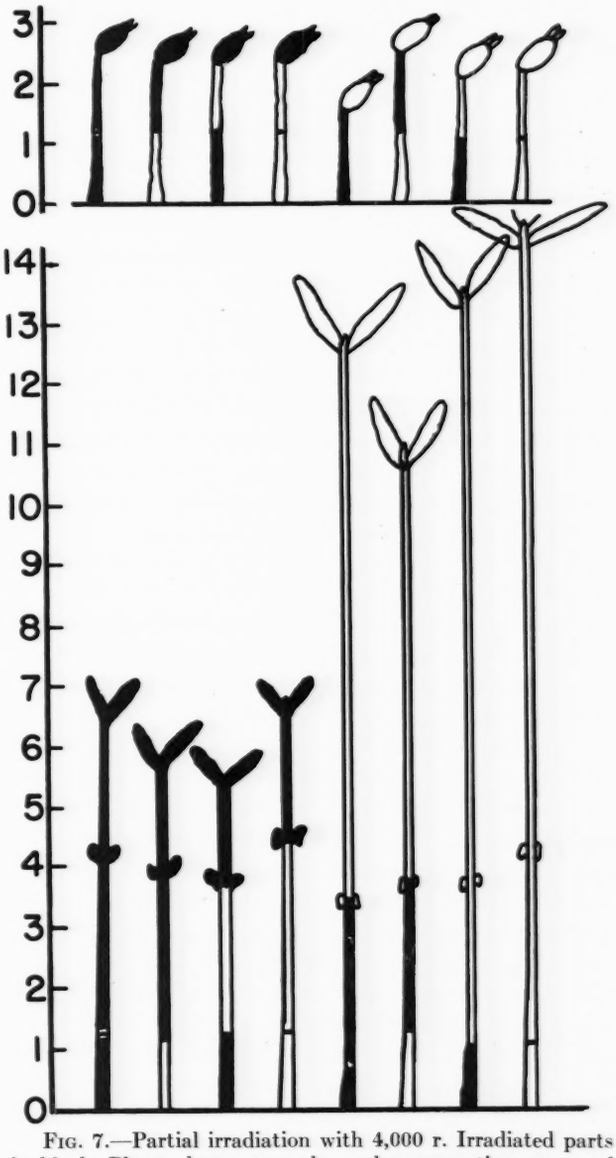

FIG. 7.—Partial irradiation with 4,000 r. Irradiated parts in black. Plants drawn to scale, each representing means of eight observations. Top row, lengths at irradiation. Bottom row; final lengths. Scales, cm.

Protoplasm is built from water, salts, and organic molecules, with energy furnished by organic molecules. The latter, in a young seedling, come from material stored in the cotyledons. Toward the end of our period of observation, some energy-rich material is contributed by the green leaves. However, it was shown, by resection of the leaves, that this contribution had little effect on longitudinal growth.

[1] The only dosage range that is of interest is that which will cause stunting on irradiation of the whole seedling. Of course, there must be some dose, probably very high, which will damage the cotyledons so much that it is equivalent to amputation.

The building blocks migrate from the cotyledons into the growing tip, where they are processed, modified, and assembled in the process which constitutes the initiation of growth. If building blocks are not processed, their flow stops; if growth initiation is curtailed by irradiation, the cotyledons remain fleshy and fail to lose their substance and to shrivel as in normal plants.

As a segment actually gets larger, its location becomes farther and farther removed from the tip, since the tip is being pushed away by the expansion of growing segments. If growth initiation and actual growth (to completion) proceed at constant rates, a steady state develops, and thus a segment at a given distance from the tip will have a characteristic growth rate and a characteristic total growth capacity (see section I).

"Growth initiation" and "growth completion" are very general terms for which one would like to substitute more specific ideas. "Cell division" and "cell stretching" might express these processes. Cell division goes on in the embryonic zone, which is located near the tip; cell stretching is located in a larger segment extending away from the embryonic zone. Cell stretching is the expression of cell differentiation, the first act of which is the initiation of growth; cell division is the process in which differentiable cells are provided. Finally, stretching is more radio-resistant than division, a finding which agrees with the fact that growth completion is more resistant than growth initiation. In spite of these very suggestive arguments, we are not prepared to substitute "cell division and stretching" for "growth initiation and completion" until a correlation is actually proved. In roots of Mung beans, a very similar situation has been studied previously. There, too, a certain portion of the growth capacity is distinguished by higher radio-resistance, and this portion was identified as growth in the process of completion by an analysis of the time course of radiation responses. The correlation between growth capacity and mitotic activity was carefully studied, both in normal and in irradiated beans, and no simple connection was found (4). We have concluded that cell division and stretching are two aspects of growth initiation and completion, but not necessarily the most important ones. Until definite evidence is obtained, we will keep using the general terms, "growth initiation and completion."

Reduction of the capacity to complete growth.—We have isolated three distinct radiation effects on the linear growth of stems in Mung bean seedlings: reduction of the capacity to initiate growth, temporary inhibition of actual growth, and reduction of the capacity to complete growth.

In normal plants the segmental elongation due to the capacity to complete growth decreases exponentially with the distance from the growing tip; in irradiated seedlings, the growth of each segment is reduced proportionally, the residual growth capacity declining exponentially with the dose. The capacity to complete growth is largely autarkic (see pp. 606 and 608). This suggests that effects on growth completion will be due to comparatively simple actions. The exponential form of the dose-response curve indicates that single events are sufficient to contribute to the total effect. What these single events are, we do not know; they must be due to the absorption of radiant energy, but this absorption could either take place in the structures concerned with growth completion, or be transmitted to them by various ways of energy transfer. If we assume direct energy absorption, then we can use the apparatus of the target theory to obtain additional information (2). Assuming, first, that the "targets" are approximately spherical, and that a single ionization constitutes a "hit," we obtain, from the slope of the dose-response curve, a target diameter of 65 mμ, or a molecular weight of about 10^8. Assuming that only the densely ionized tail-ends of the electron tracks can score a hit, we get a diameter of about 270 mμ, a molecular weight of about 10^9. The order of magnitude obtained is that of Claude's (1) particulate components of the cytoplasm. As these components are known to carry a number of enzymes, their connection with the building of protoplasm and the completing of growth is likely, and they are a plausible seat of the radiation effect which interferes with the completion of growth. This deduction, however, is based on the assumption that, in the reaction studied, the hit theory is at least approximately valid. If we assume that the effect is mediated through free radicals, then we have no means with which to estimate the size of the structures involved.

Temporary inhibition of growth.—A completely different situation is encountered when the temporary inhibition of growth is considered. Since the actual growth, at any given time, is due to growth completion rather than to growth initiation, the substrate of the effect must be growth completion. A dose of 2,500 r is sufficient to interrupt growth completion for a considerable time, and yet it has virtually no permanent effect on growth completion.

It has been shown that x-rays destroy auxin[2] (5). It is possible that this effect is responsible for the immediate temporary inhibition of growth.

[2] S. A. Gordon, personal communication.

However, it is not evident how small doses of x-rays could destroy enough auxin in the plant to cause complete cessation of growth. The auxin molecule is small, and many millions of roentgens would be required for direct destruction. However, radiant energy absorbed primarily by an atom belonging to a molecule of water or of some other substance can be transferred to the auxin molecule. By this mechanism, small doses of radiation can destroy auxin in dilute solution. In the cell, however, there are many substances which compete with the auxin molecules for the energy absorbed by the water molecules. Thus, the auxin hypothesis either implies some mechanism by which energy absorbed by water and other substances is channeled preferentially toward the auxin molecules or an extremely high affinity of auxin for free radicals.

Whether due to the destruction of auxin or to some other process, the temporary inhibition of growth must operate through mechanisms which co-ordinate the events within and possibly among cells. Otherwise, it is hard to see how the effect could be complete after comparatively small doses. In this respect, the temporary inhibition of growth completion is comparable to the temporary inhibition of mitosis which occurs after doses which are much too small to score a hit in every chromosome. The two processes are not equally radiosensitive; in bean roots, inhibition of mitosis occurs after doses which are too small to affect the growth rate (4).

Reduction of the capacity to initiate growth.—The most striking effect of irradiation is the reduction of the capacity to initiate growth. The act of initiating growth is an operation or chain of operations in which building materials (organic molecules, water, and salts) are assimilated into the living structure in such a way that they will produce an increase in size. This process could be disturbed in three ways: (*a*) by destroying the building material, (*b*) by preventing it from reaching the region where it is to be used, or (*c*) by interfering with the mechanisms of utilization (which include all intermediate metabolic processes which might be involved in the initiation of growth).

Radiation effects on the building materials are irrelevant. Salts and water are changed by irradiation, but the effect is of very short duration and could affect living structures only in the immediate neighborhood. The organic molecules which serve as building blocks are stored chiefly in the cotyledons, smaller amounts being located in the leaves and the hypocotyl. It has been shown that irradiation of these three organs has no effect on the capacity to initiate growth. Furthermore, the cotyledons are not depleted if growth fails to be initiated.

Radiation also fails to affect the transport of building materials. Salts and water are taken up by the root; irradiation of the root, even with doses which cause severe stunting, fails to affect the growth of the stem. Material from the cotyledons goes downward and upward to the growth zones; irradiation of the point of insertion of the cotyledons and other parts of the plant situated between cotyledons and growth zones does not reduce growth initiation.

Since radiation does not destroy the material needed to initiate growth and does not interfere with its transport to the region where it is used, it must act upon the mechanisms of assimilation. The effect is obtained only by irradiation of the region where growth is initiated. The dose-response curve suggests a single-hit mechanism. In dry seeds and in very young plants, a different curve is obtained, but current investigations of responses to divided doses indicate that there, too, one is dealing with a one-event reaction. This reaction is more than 20 times as radiosensitive as the reduction of the capacity to complete growth. The apparatus needed to initiate growth is much more vulnerable to irradiation than the actual process of growth. Irradiation affects some early stages of differentiation; once the process of differentiation is well under way, only high doses of radiation will seriously interfere with it.

SUMMARY

1. The observations reported concern linear growth in Mung beans—growth of individual segments and of the entire hypocotyl and the first segment of the epicotyl in normal plants, after resection of various parts, and after total or partial irradiation with various doses.

2. The growth capacity is analyzed into two components: the capacity to initiate growth, which is located in the top 2 mm, is dependent on food stored in the cotyledons, and is determined by the age of the seedling; and the capacity to complete growth, which is spread along a zone of about a 15-mm. length, is locally autarkic, and is determined by the position of the segment observed. "Growth initiation and completion" are possibly, but not necessarily, identical with "cell division and stretching."

3. Irradiation with x-rays causes three distinct effects: temporary inhibition of growth completion, and permanent reduction of the capacities of both initiating and completing growth. Permanent stunting is an effect of local irradiation.

4. An analysis of dose-response relations shows

that the apparatus for growth initiation is more vulnerable than the apparatus concerned with actual growth.

REFERENCES

1. Claude, A. Particulate Components of Cytoplasm. Cold Spring Harbor Symp., **9**:263–71, 1941.
2. Lea, D. E. Action of Radiations on Living Cells. Cambridge: At the University Press; New York: Macmillan Co., 1947.
3. Quastler, H., and Baer, M. Inhibition of Plant Growth by Irradiation. I. Discrete Steps of Inhibition and Pattern of Dose-Response Relation. J. Cell. & Comp. Physiol., **31**: 213–34, 1948.
4. Quastler, H.; Schertiger, A. M.; and Stewart, W. N. Inhibition of Plant Growth by Irradiation. IV. Linear Growth vs. Mitotic Activity. J. Cell. & Comp. Physiol. (in press).
5. Skoog, F. The Effect of X-Irradiation on Auxin and Plant Growth. J. Cell. & Comp. Physiol., **7**:227–70, 1935.

The Effect of Nitrofurazone on Growth of Fibrosarcoma in Mice*

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Nitrofurazone N.N.R., 5-nitro-2-furaldehyde semicarbazone (furacin), has been found to be effective in controlling the bacterial flora of infected wounds (10–12). Dodd (2) reported that furacin was active *in vitro*, and in some cases *in vivo*, against a large variety of gram-positive and gram-negative bacteria. Cramer (1) showed that furacin poised the oxidation-reduction potential of bacteria and suggested that it might interfere with the enzymes involved in hydrogen transport. Green (3) observed that furacin inhibited the oxidation of glucose and pyruvate in the Warburg respirometer, when bacteria were used as the source of enzymes.

Since furacin appeared to inhibit several enzyme systems of bacteria, its action on tumor growth was also investigated. In a preliminary report, Green and Friedgood (4) found that furacin retarded the growth of a transplanted fibrosarcoma in mice. In this paper, further observations of the effects of furacin on the growth and histology of this tumor will be described.

MATERIALS AND METHODS

Ninety adult inbred C3H mice of the Andervont subline were used. Equal amounts of mouse sarcoma S-13 were transplanted into the right axillary space.[1] Furacin crystals,[2] finely ground to a powder in a mortar, were suspended in peanut oil at a concentration of 200 mg/ml. This suspension was injected subcutaneously into the lumbodorsal region at a site far removed from the region of the tumor implantation. The animals were divided into six groups and treated with furacin (20 mg. in 0.1 ml. oil) according to the following treatment schedules:

Group I.—One dose injected 1 week after implantation of the tumor.

Group II.—Injected 3 days prior to transplantation, followed by another injection 1 week after transplantation.

Group III.—Injected 3 days prior to transplantation, 1 week after transplantation, and again 2 weeks after transplantation.

Group IV.—Injected 5 days before transplantation, again at the time of transplantation, and thereafter every 5 days until 25 days had elapsed, making a total of 140 mg. of furacin per mouse.

Group V.—Untreated controls.

Group VI.—Controls treated with five doses of 0.1 ml. of peanut oil at intervals of 5 days after transplantation.

The tumors were palpated daily, to note the amount of growth. The mice were weighed periodically, to determine any abnormal losses in weight. Necropsies were made at the time of death of the animals, and microscopic sections were prepared from the tumor, kidney, liver, adrenals, and gonads. All animals were fed Purina Fox Chow and housed in wire cages.

RESULTS

Table 1 compares the survival time of the tumor-bearing animals treated with furacin with that of the untreated controls. All treated animals showed an increased longevity.

The effect of furacin given only *after* tumor implantation is seen in the results with Group I. When Furacin was given *before* as well as after implantation, the greatest effects on longevity were observed. In Groups II, III, and IV, the average life span increased from 34 days to 40 days. The lower limit of longevity in each case was at least

* This work was supported by grants from Eaton Laboratories, Inc., Norwich, N.Y., Research Council, University of Missouri, and the Public Health Service.

[1] This tumor was supplied by Dr. Margaret Reed Lewis of the Wistar Institute. It was originally induced by methylcholanthrene and has since undergone transplantation through many generations with no incidence of spontaneous regression. It gives 100 per cent takes in C3H mice of the Andervont subline.

[2] The furacin used was generously supplied by Dr. L. Eugene Daily of the Eaton Laboratories, Inc., Norwich, N.Y.

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30 days, when furacin was also given *before* implantation. This result may be attributed to the 20 mg. of furacin given before transplantation. However, doses of furacin given after transplantation also had an effect, in raising the upper limit of the life span from 34 days in Group I to the maximum of 49 days in Group IV. There is not much advantage, in terms of longevity, in increasing the total dose of furacin from 60 mg. to 140 mg.

The effect of furacin was not influenced by the peanut oil in which it was suspended. The rate of tumor growth and the life span of the mice treated with peanut oil was approximately the same as that of the untreated control group.

TABLE 1

SURVIVAL TIME OF MICE AFTER IMPLANTATION OF S-13 TUMOR AND TREATMENT WITH FURACIN

Group	Number of animals	Total furacin injected* (mg.)	Average survival (days)	Range (days)	Standard deviaton
I	10	20	29	22–34	3.6
II	10	40	34	30–42	3.6
III	10	60	37	30–47	4.7
IV	30	140	40	31–49	4.7
V	15	Controls	21	18–25	2.6
VI	15	Controls plus peanut oil	22	19–27	3.4

* Details in the text.

Although furacin exhibited a definite inhibitory effect on the growth of the tumor, eventually all the animals died as a result of the malignancy. In the control series, the tumor was usually palpable within 5 days following transplantation, while in the furacin-treated mice the tumor transplant was not palpable for at least 2 weeks and grew at a correspondingly slower rate. There was no evidence of metastasis in any of the animals. The sarcoma, however, did infiltrate the surrounding tissues and the thoracic cage.

Histologic studies revealed a striking effect of furacin on the morphology of the sarcoma in treated animals. The microscopic picture of the untreated tumor was typical of a highly cellular fibrosarcoma. In sections of the furacin-treated tumor tissue there was evidence of cellular degeneration with pyknotic nuclei and a more granular cytoplasm. The tissue took an eosinophilic stain, and mitosis was greatly decreased as compared to that in the untreated controls. The microscopic differences correspond to the gross differences observed in the rate of growth.

There was no evidence of parenchymal damage to the liver or kidney. The adrenal gland, however, was hypertrophied in the furacin-treated animals. The testes and ovaries were atrophic with decreased gametogenic activity.

DISCUSSION

Relatively little is known regarding tumor chemotherapy. The "metabolite-antagonism" approach, which has been extensively applied in bacterial chemotherapy, has also been suggested for the treatment of tumors (5). Vitamin (9, 13) and purine (7) analogs have been tested against tumors.

Both the growth experiments and the histologic changes described above indicate that furacin may selectively inhibit the growth of neoplastic tissue. While a definite mechanism of action cannot be advanced at the present time, two possibilities are suggested. The first concerns the possible inhibition of glucose or pyruvate metabolism of the tumor by furacin.

Studies on bacterial metabolism with furacin (3) have shown that it inhibits the enzymes involved in the dissimilation of glucose and pyruvate. Recent experiments have shown that several nitrofurans likewise inhibit glucose dissimilation.[3] The homologous non-nitrofurans are inactive in this respect.[3]

Since members of the vitamin B complex in coenzyme form are involved in glucose and pyruvate metabolism, the following experiments not previously described may be of interest. Eight adult, albino Wistar rats were injected subcutaneously with two large doses of furacin suspended in peanut oil—200 mg. initially and the same dose again after 1 week. At the end of 2 weeks, characteristic symptoms of multiple vitamin deficiencies were observed, such as porphyrin-whiskers, rusty fur, scaly skin and feet, weakening of the hind legs, diarrhea, cheilosis, and conjunctivitis. When the administration of furacin was terminated and the animals were fed on a full diet fortified with vitamins, they returned to their original healthy state of nutrition. While no symptoms of vitamin deficiency were observed under the conditions of the tumor therapy experiments, these experiments nevertheless indicate the possibility that furacin may interfere with the carbohydrate metabolism of the tumors. However, before coming to this conclusion, appropriate experiments with the treated tumors will have to be performed.

The role of the adrenal cortex suggests another possible explanation for the mode of action of furacin on tumor growth. Lewis, Aptekman, and King (8) have demonstrated the retardation of tumors by mincing tumor grafts with adrenal

[3] Green, M. N.; Heath, E. C.; and Goldberg, H. Unpublished data.

gland tissue alone. Complete inhibition of growth of a transplanted rhabdomyosarcoma in C3H mice treated with daily injections of 1.0 mg. of cortisone has been reported by Higgins, Woods, and Bennett (6). Since hypertrophy of the adrenal cortex was observed in the treated animals, furacin may exert its effect on tumor growth indirectly through the adrenal gland. Investigations now being conducted on adrenalectomized mice should clarify this possibility. The observed atrophy of the testes and ovaries following prolonged furacin therapy may also be related to the enlargement of the adrenal cortex.

SUMMARY

Furacin suspended in peanut oil, injected subcutaneously, retarded the growth of fibrosarcoma S-13 in C3H mice of the Andervont subline.The life span of the mice was approximately doubled by this treatment (from 21 to 40 days). Microscopic examination of the furacin-treated tumors revealed increased cellular degeneration, with lessened mitotic activity. Hypertrophy of the adrenal cortex and atrophy of the testes and ovaries have also been observed in the furacin-treated animals.

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REFERENCES

1. CRAMER, D. L. The Mode of Action of Nitrofuran Compounds. II. Application of Physiochemical Methods to the Study of Action against *Staphylococcus aureus*. J. Bact., **54**:119–25, 1947.
2. DODD, M. C. The Chemotherapeutic Properties of 5-Nitro-2-Furaldehyde Semicarbazone (Furacin). J. Pharmacol. & Exper. Therap. **86**:311–23, 1946.
3. GREEN, M. N. The Effect of Furacin (5-Nitro-2-Furaldehyde Semicarbazone) on the Metabolism of Bacteria. Arch. Biochem., **19**:397–406, 1948.
4. GREEN, M. N., and FRIEDGOOD, C. E. Action of Furacin in Delaying Growth of a Transplanted Fibrosarcoma in Mice. Proc. Soc. Exper. Biol. & Med., **69**:603–4, 1948.
5. GREENBERG, D. M., and SCHULMAN, M. P. Application of "Metabolite Antagonism" to Cancer Research. Science, **106**:271–72, 1947.
6. HIGGINS, M.; WOODS, K. A.; and BENNETT, W. A. The Influence of Cortisone (Compound E) upon the Growth of a Transplanted Rhabdomyosarcoma in C3H Mice. Cancer Research, **10**:203, 1950.
7. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**: 511–14, 1949.
8. LEWIS, M. R.; APTEKMAN, P. M.; and KING, H. D. Retarding Action of Adrenal Gland on Growth of Sarcoma Grafts in Rats. J. Immunol., **61**:315–19, 1948.
9. MOORE, A. P.; STOCK, C. C.; SUGIURA, K.; and RHOADS, C. P. Inhibition of Development of Sarcoma 180 by 4-Amino-N^{10}-Methyl Pteroylglutamic Acid. Proc. Soc. Exper. Biol. & Med., **70**:396–98, 1949.
10. NETER, E., and LAMBERTI, T. G. Hemolytic Streptococcus in Wounds of Rabbits. Action of 5-Nitro-2-Furaldehyde Semicarbazone. Am. J. Surg., **72**:246–47, 1946.
11. SHIPLEY, E. R., and DODD, M. D. Clinical Observations on Furacin Soluble Dressing in the Treatment of Surface Infections. Surg., Gynec. & Obst., **84**:366–72, 1947.
12. SNYDER, M. L.; KIEHN, C. L.; and CHRISTOPHERSON, J. W. Effectiveness of a Nitrofuran in the Treatment of Infected Wounds. Mil. Surgeon, **97**:380–84, 1945.
13. SUGIURA, K.; MOORE, A. C.; and STOCK, C.C. The Effects of Aminopterin on the Growth of Carcinoma, Sarcoma, and Melanoma in Animals. Cancer, **2**:491–501, 1949.

Synthesis of 2-Acetylaminofluorene-9-C^{14} and 2-Acetylaminofluorene-ω-C^{14}*†

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A study of the metabolism of 2-acetylaminofluorene (AAF) might be expected to throw considerable light on chemical carcinogenesis. Preliminary work by Morris and Westfall (4) was able to account for only 32 per cent of the ingested dose. The colorimetric method used depended on the presence of a diazotizable amino group. Because of the transformations that such a group can undergo we decided to incorporate carbon 14 into the 9-position of AAF. In this location it is likely that the carbon would be separated only with difficulty from the aromatic part of the molecule. A preliminary announcement of this synthesis was made in 1949 by the present authors (5). The details are given now because of the widespread interest in AAF.

It seems probable that the acetyl group is early removed in the metabolism of AAF, because Morris, Dubnik, Dunn, and Johnson (3) found that both 2-aminofluorene and 2-nitrofluorene are carcinogenic. We have, therefore, also synthesized AAF with carbon 14 in the methyl group of the side chain.

If the premises stated above are correct, then radioactive carbon dioxide should be found in the expired air from animals fed AAF with the radioactive side chain but not from those fed AAF with the radioactivity in the fluorene nucleus.

EXPERIMENTAL

PREPARATION OF 2-ACETYLAMINOFLUORENE-9-C^{14}

Figure 1 gives an outline of the reactions employed. The asterisks denote radioactive carbon 14. The percentages refer to yields, and the c/m refers to counts per minute using a Geiger tube with a window of 4–5 mg/cm² at a distance of $1\frac{1}{8}$ inches, connected to a Higginbotham-type scaler. All counts were for samples 22 mg/cm².

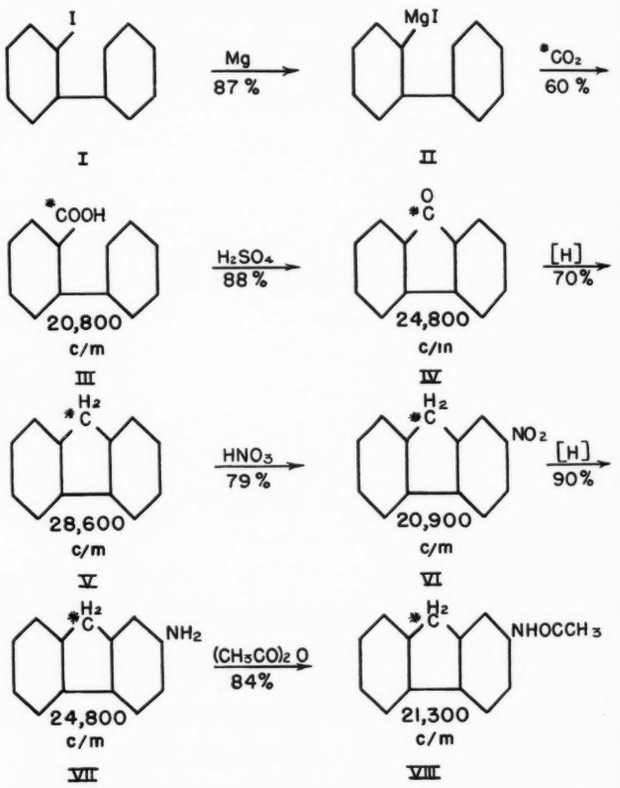

FIG. 1.—Outline of reactions employed in the synthesis of 2-acetylaminofluorene-9-C^{14}.

Biphenyl-2-carboxylic acid-C^{14}, III.—The magnesium (1 gm.) and 20 ml. of ether were placed in a 75-ml. Erlenmeyer flask fitted with a reflux condenser carrying at the top a dropping funnel and a calcium chloride drying tube. The dropping funnel contained 20 ml. of ether and 2-iodobiphenyl (11.5 gm.). The ether solution of iodobiphenyl was dropped slowly down the reflux condenser into the refluxing ether. Once started, the reaction proceeded vigorously without the aid of heat. After the reaction had subsided, heat was again applied

* Experiments on the metabolism of these compounds in the rat are reported by Morris, Weisburger, and Weisburger, Cancer Research, **10**:616–20, 1950.

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Thanks are due to Dr. Charles D. Stevens for his assistance.

† The nomenclature of these compounds (5) has been revised in conformity with the policy of *Cancer Research*.

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for ½ hour to drive the reaction further toward completion. A titration of the Grignard reagent revealed an 84 per cent yield.

Carbonation was the most critical reaction of the series of steps in the total synthesis. This was the case because (*a*) danger was involved in possible inhalation of the radioactive carbon dioxide, (*b*) the radioactive carbon must be incorporated with the highest efficiency, and (*c*) the apparatus employed required careful manipulation for proper functioning.

Reference to Figure 2 will be made in describing the procedure of carbonation of the Grignard reagent.

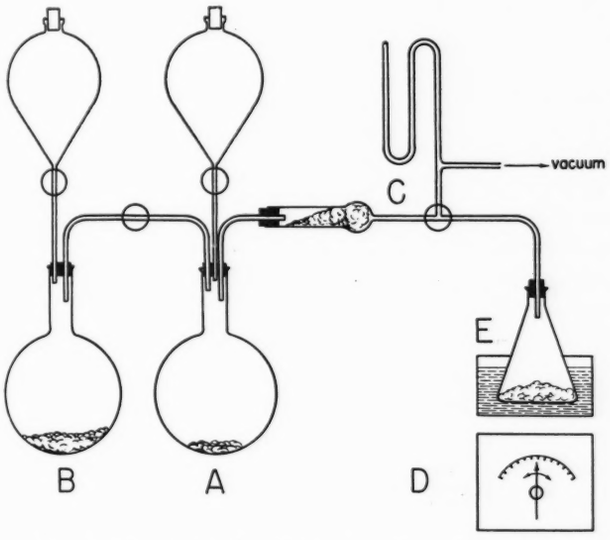

FIG. 2.—Apparatus for the carbonation of the Grignard reaction.

The 125-ml. Erlenmeyer flask, E, which contained the Grignard reagent, was cooled to —70° C. with a surrounding methanol dry ice bath. This reduction in temperature caused the contents of flask E to solidify to a white paste. The valve between flasks A and B was opened as well as the 3-way valve at C. The latter connected both sides of the apparatus to the manometer shown in the figure and to a Hyvac pump which is not shown. The system was then evacuated to 3 mm., after which the valve at C was turned so that both sides of the system were open to each other, but were closed off from the pump. The stopcock between flasks A and B was also closed at this point.

In order to best utilize the radioactive carbon, isotopic carbon dioxide was first generated by dropping phosphoric acid on pure radioactive barium carbonate[1] (0.394 gm., 2 mc.) contained in flask A. Flask A was then heated gently to complete the liberation of $C^{14}O_2$, and the well cooled (with dry ice) Grignard reagent in flask E was stirred by magnetic stirrer, D.

After several minutes, phosphoric acid was allowed to enter flask B, which held ordinary barium carbonate (5.7 gm.), and the stopcock between A and B was opened. This swept out any radioactive carbon dioxide remaining in the system and also furnished enough carbon dioxide to complete the reaction with the remaining Grignard reagent in flask E. It took approximately twenty minutes for all the carbon dioxide to be expelled from flask B with cautious heating, and during this period the magnetic stirrer operated continually. The valve at C was turned, closing off the right side of the system from the rest of the apparatus, and the flask containing the carbonated Grignard reagent slowly came to room temperature.

After standing overnight, the reaction mixture contained two layers, an upper ether layer and a lower viscous layer, which was the Grignard addition complex. This lower layer was hydrolyzed slowly with dilute hydrochloric acid, forming radioactive biphenyl-2-carboxylic acid, which dissolved in the ether. The ether solution was separated, and the remaining water layer extracted 3 times with 10-ml. portions of ether to recover any product in the water. These ether extractions were added to the original ether solution and the whole evaporated, leaving a pink solid of biphenyl-2-carboxylic acid contaminated with biphenyl and 2-iodobiphenyl.

Heating to boiling with dilute aqueous potassium hydroxide formed the potassium salt of the acid, which proved quite soluble in water. Since the impurities were insoluble in water, filtration of the hot solution removed them. Extracting the material on the filter paper with more dilute potassium hydroxide and washing with warm water insured a complete recovery of the acid salt. Acidification of the filtrate precipitated the free acid. By again forming the potassium salt, filtering, and reacidifying, a pure compound, melting at 114° C., was obtained.

In order to get a maximum yield of acid based on the carbon dioxide employed, the proportions set out previously are mandatory. A delicate balance between the amount of Grignard reagent and the quantity of carbon dioxide was found to exist. Our greatest difficulty, however, was in getting enough carbon dioxide absorbed in the ether to fully react with the Grignard reagent. In most previous carbonations of this type, a large amount of ether was employed, as it was felt that this would allow for a greater absorption of carbon

[1] $BaC^{14}O_3$ was obtained from the Oak Ridge National Laboratories under allocation from the U.S. Atomic Energy Commission.

dioxide. It was found, however, that a small amount of ether must be used, i.e., just enough to allow for stirring of the Grignard paste when cold. In this form, carbon dioxide was rapidly taken up. The yield and activity are given in Figure 1.

Fluorenone-9-C^{14}, IV.—The biphenyl-2-carboxylic acid-C^{14} (3.6 gm.) was placed in a 50-ml. Erlenmeyer flask containing the sulfuric acid (18.4 gm.) and surrounded by a water bath. The temperature of the bath was rapidly brought to 85° C. and was held at that point for 25 minutes. Pouring the contents of the flask into ice and water precipitated 3 gm. of the yellow fluorenone.

The yellow material was made alkaline with aqueous sodium hydroxide and warmed to 75° C. After cooling well with ice, the fluorenone was filtered off, leaving the sodium salt of any unreacted biphenyl carboxylic acid in the filtrate. Acidification of the filtrate, however, gave none of the acid.

Fluorene-9-C^{14}, V.—The fluorenone obtained in the preceding section was reduced to fluorene with zinc amalgam, prepared and used according to Martin's (2) directions.

Zinc amalgam (75 gm.), water (9 ml.), and hydrochloric acid (21 ml.) were refluxed; and small portions of fluorenone, dissolved in ethanol (75 ml.), and hydrochloric acid were added alternately. Refluxing and stirring were continued for an additional 7 hours, and after this time most of the yellow color of the fluorenone had disappeared.

Water was added to the decanted solution until the fluorene was completely precipitated. There was obtained 1.9 gm. of slightly impure fluorene that melted at 109°–111° C. This was used without purification for the next step.

2-Nitrofluorene-9-C^{14}, VI.—The fluorene obtained as described above was dissolved in glacial acetic acid (17 ml.) and nitrated substantially as described in Organic Syntheses (1). The product was collected in a Gooch crucible and dried. The light yellow compound melted at 155°–156° C. The yield was 1.7 gm.

2-Aminofluorene-9-C^{14}, VII.—Ethanol (46.7 ml.), water (10.3 ml.), and calcium chloride (0.6 gm.) were placed in a reflux apparatus, and nitrofluorene (1.7 gm.) and zinc dust (5.7 gm.) were added. The mixture was boiled vigorously for 5 hours (1), filtered, and poured into water (114 ml.). After standing overnight, the flocculent white precipitate was filtered and dried; the melting point was 125°–126° C., the yield, 1.3 gm.

2-Acetylaminofluorene-9-C^{14}, VIII.—The 2-aminofluorene (1.3 gm.) was dissolved in boiling benzene (7.5 ml.), and acetic anhydride (0.82 gm.) was dropped slowly into the refluxing mixture.

The crude 2-acetylaminofluorene that precipitated was dissolved in 12 ml. of ethanol, heated to boiling, and enough water was added to almost bring about a turbidity at the boiling point. Gradual cooling resulted in a pure white precipitate melting at 194°–195° C.; the yield was 1 gm.

Preparation of 2-Acetylaminofluorene-ω-C^{14}

Acetyl chloride-2-C^{14}.—Radioactive sodium acetate with carbon 14 in the 2-position was obtained[2] in two 25-ml. sealed flasks, each containing 0.5 mc. These flasks were cut off 1 inch from the rounded portion and attached at B, Figure 3. First, however, a glass-inclosed metal bar and a thin capsule containing base sodium acetate (1.5 gm.) had been placed in each flask.

A cloth bag filled with crushed dry ice was placed on flask C, and the water bath at B heated to 55° C. Then, a few drops of phosphorus oxychloride (total 4.6 gm.) contained in the dropping funnel A were allowed to enter the system and react with the radioactive sodium acetate. A vigorous whipping of the magnetic stirring bar soon broke the thin glass capsule containing the inactive sodium acetate, and more phosphorus oxychloride was allowed to enter flask B. This resulted in a vigorous reaction producing ordinary acetyl chloride, which served to sweep the already formed radioactive acetyl chloride into flask C. In this way it was felt that a maximum amount of the radioactive acetyl chloride was obtained. The product boiling between 50° and 55° C. was collected.

A second reaction identical to the one previously described was carried out. Consequently, all of the acetyl chloride was collected in flask C. A yield of 70.1 per cent of acetyl chloride was consistently obtained in a number of trial runs prior to the radioactive run. As a result one might assume that the yield when using radioactive material should be the same, and it was accordingly listed as such. However, some difficulty was encountered in reacting the phosphorus oxychloride with the radioactive sodium acetate, as the latter firmly adhered to the bottom of the flask. Consequently, more heat was applied to the radioactive run than in the trial runs. It is felt that a better reaction could have been accomplished by first breaking up the adhering sodium acetate with chips of glass.

2-Acetylaminofluorene-ω-C^{14}.—Dry sodium bicarbonate (2.0 gm.), benzene (40.0 ml.), and 2-aminofluorene (4.6 gm.) were placed in a 125-ml. Erlenmeyer flask, D, Figure 3. On gently refluxing, the aminofluorene dissolved, and the sodium bicarbonate was finely dispersed. Stopcock C was then opened and the radioactive acetyl chloride

[2] From the Tracerlab.

allowed to run through the condenser into flask D. An immediate precipitate of 2-acetylaminofluorene-ω-C^{14} resulted, but refluxing was continued for ½ hour. The flask was cooled well with ice, and the AAF was filtered and dried. By adding ligroin to the filtrate, a small amount precipitated and was recovered.

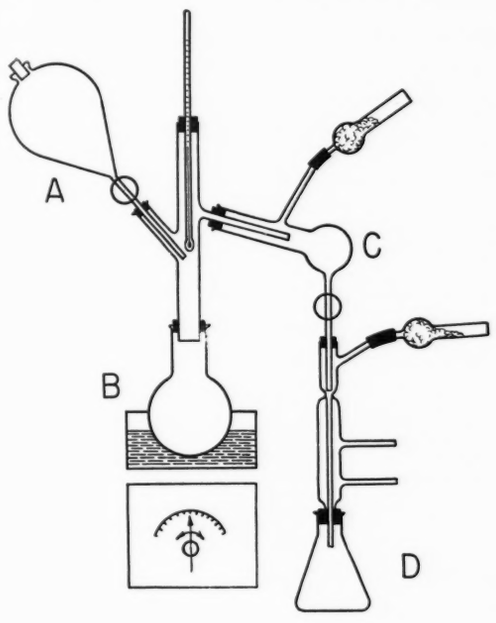

FIG. 3.—Apparatus for the preparation of acetyl chloride-2-C^{14}.

The melting point of the precipitate was 216°–220° C., which indicated principally impure 2-aminofluorene (AF) hydrochloride. Consequently, the solid was extracted with hot water to remove amine hydrochloride and inorganic materials. The m.p. was then 150°–165° C., indicating impure AAF. Pure AAF melts at 194° C. A second extraction failed to improve the melting point, and therefore the precipitate was recrystallized from aqueous ethanol. This resulted in m.p. 172°–178° C. A second recrystallization failed to alter the melting point. It was felt that an equilibrium between AF and AAF existed. To shift this equilibrium, 0.3 gm. of pure inactive 2-acetylaminofluorene was added, and recrystallization with aqueous ethanol now gave the pure compound, m.p. 194° C. The yield was 2.3 gm. or 41.1 per cent. The low yield resulted from the repeated recrystallizations, although some loss of the volatile acetyl chloride-2-C^{14} may have occurred. In repeating the work it would be advisable to add the inactive acetylaminofluorene before the first recrystallization. A sample containing 22 mg/cm² gave 10,600 counts per minute under the same conditions used for counting the other compounds.

SUMMARY

The synthesis of the following radioactive derivatives of 2-acetylaminofluorene has been described in detail:

2-Acetylaminofluorene-9-C^{14}
2-Acetylaminofluorene-ω-C^{14}

REFERENCES

1. KUHN, W. E. 2-Nitrofluorene and 2-Aminofluorene. In BLATT, A. H. (ed.), Organic Syntheses, **2**:447–48, 1943. New York: John Wiley & Sons.
2. MARTIN, E. L. A Modification of the Clemmensen Method of Reduction. J. Am. Chem. Soc., **58**:1438, 1936.
3. MORRIS, H. P.; DUBNIK, C. S.; DUNN, T. B.; and JOHNSON, J. M. Tumors Produced in Rats after Ingestion or Painting of 2-Nitro-, 2-Amino-, N-Acetyl-2-Amino-, and N-Diacetyl-2-Aminofluorene. Cancer Research, **7**:730–31, 1947.
4. MORRIS, H. P., and WESTFALL, B. B. Distribution of N-Acetylaminofluorene in the Rat Following a Single Feeding. J. Nat. Cancer Inst., **9**:149–54, 1948.
5. RAY, F. E., and GEISER, R. C. 2-Acetylamino-9-C^{14}-Fluorene. Science, **109**:200–201, 1949.

The Distribution of Radioactivity following the Feeding of Carbon 14-labeled 2-Acetylaminofluorene to Rats*†

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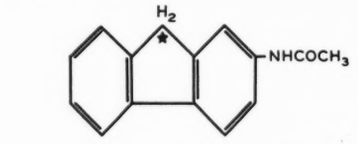
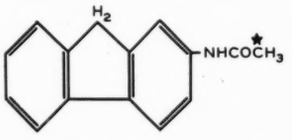
INTRODUCTION

2-Acetylaminofluorene (AAF) is at present one of the most outstanding examples of a class of chemical carcinogens which, when fed to rats, induces tumors in a variety of organs and tissues. Studies of the distribution and excretion of AAF have been reported by Morris and Westfall (7). The tissues and excreta in their experiments were extracted with acetone, and the extracts were heated with dilute hydrochloric acid to hydrolyze any AAF or its complexes to 2-aminofluorene (AF). The AF was then diazotized and coupled with R-salt to form a dye which was estimated colorimetrically. This method requires the presence of the aromatic amino group. Morris and Westfall could account, however, for only about one-third of the dose administered, indicating that an appreciable amount of the ingested AAF was transformed into metabolites which did not possess the aromatic amino group, or in which the group was firmly bound in complex linkages. The occurrence of chemical reactions of AF with simple body constituents such as pyruvic acid, as shown by Neish (8), also seems possible. Since it is not known whether AF or some metabolite functions as the primary carcinogen, it is of importance to be able to ascertain first the location of all compounds resulting from the metabolism of AAF.

One excellent method for this purpose is to study the metabolic path of the carcinogen, labeled with C^{14} at a stable point in the molecule which would not be readily broken down during metabolism. Using radioactive tracer technics it would thus be possible to follow the administered material independently of possible transformations occurring in the side chain or in other parts of the molecule. AAF with carbon 14 in the 9-position was synthesized by Ray and Geiser (9, 10) for such experiments. Furthermore, in order to investigate the eventual role played by the acetyl group, the distribution of radioactivity following administration of the carcinogen with carbon 14 in the methyl group or ω-position of the acetylamino side chain (10) was also studied.



2-ACETYLAMINOFLUORENE – 9 – C^{14}



2-ACETYLAMINOFLUORENE – ω – C^{14}

It was thought that a study of the distribution of radioactivity from the labeled carcinogen might also make it possible to determine whether the incidence of distant tumors could be correlated with the relative concentration of a radioactive compound at certain sites in the body. This report gives some of the initial results thus far obtained in a series of studies on the distribution and metabolism of AAF now in progress in this laboratory.

PROCEDURE

Young Buffalo (6) rats weighing between 77 and 133 gm. were shaved in order to avoid subsequent processing of the hair. Either one or the other labeled compound was administered by stomach tube in a propylene glycol solution at a

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† The radio carbon used in this investigation was obtained on allocation from the United States Atomic Energy Commission after being synthesized into 2-acetylaminofluorene by F. E. Ray and C. R. Geiser.

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dose of 25 mg/100 gm of body weight. This procedure provided for giving each rat a comparable dose based on the weight of the animal, and it paralleled as closely as possible the usual route of administration of the compound in tumor-producing experiments. Propylene glycol was used as a vehicle, because AAF is more soluble in it than in a neutral fat such as coconut oil, thus permitting the administration of a greater amount of radioactivity in a smaller volume of vehicle. In addition, it was hoped that the miscibility of propylene glycol with water would result in more rapid absorption, which was of importance in studying the distribution of the radioactivity during short-term experiments.

The animal was placed in a metabolism cage (large desiccator, Corning No. 3120, 250 mm. in diameter, with air in- and outlets), so designed that feces, urine, and respired air could be collected separately (3). A stream of carbon dioxide-free air was drawn through the chamber and absorbers at the maximum rate allowable by the latter (approximately 20 l/hour), and the respired CO_2 was collected by hourly increments in 75 cc. of a 1.5 per cent solution of NaOH. In one experiment air was drawn through the system for 1 hour after removal of the rat; a negligible amount of $BaCO_3$ was obtained upon precipitation. After the desired length of time the animal was removed from the metabolism cage; care was taken that any urine excreted as a result of handling of the animal was collected. The heart of the anesthetized animal was then punctured, and as much blood as possible was withdrawn. The animal was dissected immediately, and the organs were minced, dried *in vacuo* at room temperature for 24–48 hours, and weighed. The carcass contained in a beaker was cooked in a stainless steel pressure cooker at 15-lb. pressure for 90 minutes. The cooking facilitated the quantitative separation of the bones from the muscle and other soft tissue. The process of separating the bones from the cooked carcass resulted in division of the carcass into small fragments. These were combined with the broth and dried *in vacuo* for 4–6 days and then ground in a mortar. In the meantime, the dried bones were broken into small fragments, the bone fat extracted with ether, and the organs, tissues, and bones ground to a powder in a mortar. Suitable aliquots were burned in duplicate by the wet combustion method of Van Slyke *et al.* (11) in a modified Lindenbaum-Schubert-Armstrong (5) apparatus. The resulting duplicate samples of barium carbonate were counted[1] separately with a windowless gas-flow type counter (Nucleometer), and the activity expressed as the average count of the two samples. Four to six aliquots of the carcass were burned in order to reduce the sampling error. The specific activity of the carcass was low, but since it represented a very large portion of the entire rat, it was essential to have a very accurate carcass count to avoid the multiplication error.

[1] We are grateful to Dr. J. C. Reid for the use of his counting equipment.

RESULTS

The distribution of the radioactivity[2] in rat No. 1, 2 hours after the administration of 2-acetylaminofluorene-9-C^{14}, is presented in detail in Table 1. As might be expected, most of the activity remained in the gastro-intestinal tract. Sizable amounts, however, were found in the liver, kidneys, and urine. The amount of radioactivity found in the respiratory carbon dioxide was almost negligible. Two hours after feeding, 82 per cent of the dose still remained in the alimentary canal, of which 68 per cent was in the stomach; a slight amount was detected in the caecum, while 2.5 per cent had been excreted in the urine. The carcass fraction accounted for 10 per cent of the total.

Small, but detectable, amounts of radioactivity were found in all other organs and tissues examined for activity except the pituitary gland. The amount of activity shown in these organs compared to the total activity is slight, but nevertheless it does indicate where some of the activity, and, presumably, the AAF or its metabolic products, are to be found as early as 2 hours after ingestion. Bones, bone fat, and brain had the highest activity; heart, pancreas, lungs, and testes were next, while blood, spleen, bladder, eyeballs, Harderian and salivary glands, thymus, thyroid, and adrenals each showed less than 0.01 per cent of the activity.

It can be concluded from these data that there is a very rapid absorption of a considerable quantity of the radioactivity administered as AAF-9-C^{14} and that there is a wide distribution of detectable quantities of radioactive material throughout the body of the rat within a period of 2 hours following ingestion.

The same general distribution of radioactivity was found in another experiment 6 hours after administration of radioactive AAF-9-C^{14}. Less was found, however, in the liver and a higher percentage in the kidney and urine than was found 2 hours after administration, showing that urinary

[2] Activity as used herein, unless otherwise indicated, refers to the percentage of the total activity recovered. The recovered activity ranged from 95 to 98 per cent of the administered activity. Therefore, the data can be regarded as also showing essentially the percentage distribution of administered activity.

excretion occurs rapidly (Table 1, rat No. 5). As in the previous experiment, the activity of the respiratory carbon dioxide was negligible. The Harderian gland had a relatively high specific activity. The data are summarized graphically for rat No. 5 by the solid bars in Figure 1. Approximately 88 per cent of the activity was found in the alimentary tract 6 hours after administration, slightly more in the stomach than at 2 hours, less in the small intestine, but more in the caecum and urine, while there was less activity in the carcass. The liver activity was less at 6 hours than at 2 hours, but the activity of the kidneys was higher.

Of the remaining organs and tissues, blood, bones, and bone fat contained the most activity; the heart, pancreas, lungs, brain, Harderian and salivary glands were intermediate, and the spleen, bladder, ovaries, uterus, eyeballs, thyroid, and adrenals contained the least activity. No measurable activity was found in the pituitary or in the body fat.

AAF-ω-C^{14} was administered in a third experiment, and a necropsy was performed on the animal 6 hours later. A fairly widespread distribution of appreciable amounts of radioactivity was found, as shown in detail by the data for rat No. 3 in Table 1. Six per cent of the total activity was recovered in the respired carbon dioxide, whereas no appreciable amount was recovered 6 hours after AAF-9-C^{14} was administered. The 6-hour urine, on the other hand, was less active after administration of the side chain-labeled compound than

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN THE RAT AFTER ORAL ADMINISTRATION OF A PROPYLENE GLYCOL SOLUTION OF C^{14}-LABELED 2-ACETYLAMINOFLUORENE

	RAT NO. 1, WT. 77 GM., 2 HRS. 1.60 ML. SOLUTION 19.31 MG. AAF-9-C^{14}			RAT NO. 5, WT. 110 GM., 6 HRS. 1.10 ML. SOLUTION 27.45 MG. AAF-9-C^{14}			RAT NO. 3, WT. 133 GM., 6 HRS. 2.78 ML. SOLUTION 33.39 MG. AAF-ω-C^{14}		
	Total c./min	c./min/mg dry tissue	Per cent total recovered c.	Total c./min	c./min/mg dry tissue	Per cent total recovered c.	Total c./min	c./min/mg dry tissue	Per cent total recovered c.
Stomach*	1,410,000	896	67.5	2,320,000	9,941	75.3	106,000	545	5.52
Small intestine*	298,000	240	14.3	213,000	197.0	6.91	1,130,000	865	58.9
Caecum*	3,740	10.2	0.18	169,000	293	5.49	4,000	7.1	0.21
Large intestine*	3,530	22.9	0.17	516	2.5	0.02	4,590	18.1	0.24
Respiratory CO_2	140	0.15	0.007	510	0.07	0.02	116,000	23.0	6.04
Urine	47,300	6.0†	2.26	207,000	1,529	6.72	23,200	10.8†	1.21
Feces	219	18.4	0.01						
Liver	45,900	32.9	2.20	21,500	13.7	0.7	122,000	59.4	6.35
Kidney	10,900	46.4	0.52	34,100	114	1.11	12,500	36.9	0.65
Lungs	5,400	32.8	0.26	377	2.8	0.01	4,670	27.9	0.24
Bladder	1,640	90.4	0.08	98	5.5	0.003	3,220	31.5	0.17
Uterus				77	2.2	0.002	4,420	27.0	0.23
Embryos							2,080	22.3	0.11
Heart	2,560	21.1	0.12	578	4.57	0.02	2,900	18.5	0.15
Blood	1,480	16.6	0.07	3,040	6.47	0.1	3,240	10.1	0.17
Spleen	1,260	26.9	0.06	146	2.44	0.005	2,030	26.5	0.11
Eyeballs	231	7.3	0.01	77	2.0	0.002	370	0.8	0.02
Brain	11,700	32.3	0.56	643	2.2	0.02	15,700	45.9	0.82
Bones (defatted)	8,440	3.7	0.40	2,220	0.44	0.07	22,200	4.4	1.16
Bone fat	9,730	27.0	0.47	2,600	3.34	0.08	3,590	5.7	0.18
Abdominal fat				0	0	0.0	42,400	11.3	2.21
Pancreas	4,600	31.1	0.22	720	2.21	0.02	4,600	30.9	0.24
Ovaries				93	2.9	0.003	776	16.3	0.04
Testes	4,800	16.8	0.23						
Thymus	813	18.6	0.04	161	2.47	0.005	1,600	10.4	0.08
Thyroid	48	13.3	0.002	11	4.7	0.0003	43	33.1	0.002
Pituitary	0	0	0.0	0	0	0.0	55	34.3	0.003
Adrenals	346	33.9	0.02	37	1.3	0.001	391	21.1	0.02
Harderian glands	1,580	34.2	0.08	436	7.5	0.014	14,800	707.0	0.77
Salivary glands	1,900	23.1	0.09	253	2.2	0.008	4,530	32.9	0.24
Carcass	210,000	13.6	10.1	103,000	3.8	3.34	269,000	8.6	14.0
TOTAL RECOVERED COUNTS:	2,090,000‡			3,080,000§			1,920,000‡		
TOTAL ADMINISTERED COUNTS:	2,200,000‡			3,220,000§			1,950,000‡		
RECOVERY:	95			96			98		

* And contents.

† As collected, not dried.

‡ 1 count/min = 3.09 disintegrations/min.

§ 1 count/min = 2.99 disintegrations/min.

after the 9-C^{14} compound. Many of the organs and tissues of the rat which received the acetyl-labeled AAF contained several times the amount of radioactivity found with the AAF-9-C^{14}, as contrasted graphically by the data in Figure 1. Much more activity from the acetyl-labeled AAF was also found in the small intestines plus contents, in the liver, carcass, and the total for all other organs and tissues, while less activity was present in the kidney and caecum and contents.

The bones, body fat, bone fat, brain, blood, uterus, and Harderian and salivary glands had higher activity 6 hours after the acetyl-labeled compound was given than after administering the AAF-9-C^{14}. The data definitely show a different distribution of activity between the 9-C^{14} and the acetyl-labeled AAF.

The influence of stomach contents upon absorption of AAF was disclosed in one experiment. At necropsy the stomach of this rat was full of paper toweling and food, which the animal had apparently eaten during the night prior to administration of the carcinogen. Most of the activity still remained in the stomach 6 hours after administration; very little had been distributed in the organs, although somewhat more had been excreted in the urine than was excreted by the 6-hour rat given the acetyl-labeled AAF.

DISCUSSION

The results with the 9-labeled AAF showed that excretion took place rapidly, beginning within 2 hours after administration. The negligible amount of radioactivity in the respired carbon dioxide up to 6 hours indicates that the metabolic transformations do not oxidize the 9-carbon atom to carbon dioxide to any appreciable extent during this period.

The excretion in the breath of 6 per cent of the radioactivity from the ω-labeled AAF within 6 hours, compared to only a trace found in the rat given the AAF-9-C^{14}, points to an outstanding difference in the distribution of activity between the 9-labeled and the ω-labeled AAF. The acetyl group apparently was split off from the rest of the molecule and was probably incorporated into the body acetate pool, where it was, in part at least, rapidly metabolized to carbon dioxide. While Wilson *et al.* (12) and Bielschowsky (1) infer that the acetyl group is readily removed by hydrolysis *in vivo*, it is believed these experiments furnish direct proof of its occurrence.

The contrast between the distribution of radioactivity following feeding of 9-labeled and the ω-labeled AAF is brought out in Figure 1. The relatively higher radioactivity of the body fat in the rat given the latter compound may also signify that some of the acetate originating from the hydrolysis of the side chain is incorporated into adipose tissue.

The further observation, at 6 hours, that more activity was present in the urine and less in several other organs and tissues when AAF-9-C^{14} than when AAF-ω-C^{14} was administered may permit speculation concerning a possible mechanism involved. If it is assumed that deacetylation is the first step in the metabolism of AAF, followed subsequently by different pathways of metabolism of the two fragments, one might expect the acetyl group to enter the body acetate pool and be distributed more generally in the organs and tissues with less excreted in the urine. On the other hand, that portion of the molecule containing the aromatic nucleus might be excreted in the bile with reabsorption occurring from the intestinal tract and subsequent excretion of considerable amounts in the urine.

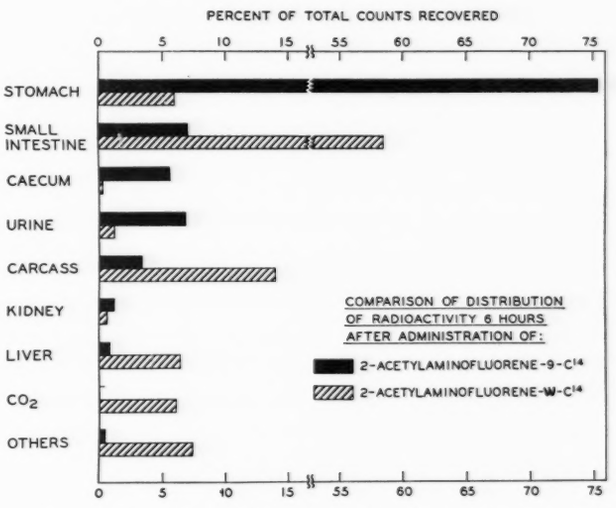

FIG. 1.—The distribution of radioactivity 6 hours after administration of AAF-9- and -ω-C^{14}.

An explanation for the induction of some of the AAF-induced distant tumors is suggested by the high specific activity of the Harderian gland. This might be related to the occurrence of eye tumors (2, 6). It seems likely that the presence of high concentrations of AAF or some metabolite in certain tissues or organs only a few hours after ingestion may be the inciting factors for carcinogenesis therein, especially if a high level of the compound is maintained at such sites during prolonged feeding.

The widespread distribution of radioactivity, as an indicator of the carcinogen or its metabolic products, might explain the appearance of tumors in many parts of the body. This can be opposed

to a locally acting carcinogen such as 1,2,5,6-dibenzanthracene, which is found to be excreted very rapidly through the liver-bile-intestinal tract and feces (4). An appreciable amount remains at the site of administration only when the material is injected subcutaneously.

Although there is at present no explanation for the apparently higher concentration of radioactivity in many tissues of the 2-hour as compared to the 6-hour rat receiving the 9-C^{14}-labeled compound, the findings seem to corroborate those of Morris and Westfall (7), who found, by means of a chemical method, maxima of concentration of AAF in the first few hours after administration.

SUMMARY

The distribution of radioactivity was studied in rats following oral administration of a single dose of 2-acetylaminofluorene-9-C^{14} at 2 and 6 hours, and 2-acetylaminofluorene-ω-C^{14} at 6 hours.

A considerable quantity of the administered radioactivity of 2-acetylaminofluorene-9-C^{14} was absorbed within 2 hours, with detectable quantities present throughout the body of the rat. A wide distribution of radioactivity was also noted at 6 hours, after administration of either compound. However, the path taken by the radioactivity of the 9-labeled compound was different from that of the methyl-labeled derivative. Less of the latter was excreted in the urine, and more was found in the organs and tissues.

Six per cent of the administered radioactivity from 2-acetylaminofluorene-ω-C^{14} was eliminated in the breath within 6 hours, while negligible amounts of radioactivity from the 9-C^{14}-labeled compound were expired during the same period, which supports the view that the acetyl group of 2-acetylaminofluorene is split off by the rat, at least in part, and metabolized independently of the remainder of the molecule.

REFERENCES

1. Bielschowsky, F. The Carcinogenic Action of 2-Acetylaminofluorene and Related Compounds. British M. Bull., **4**:382–85, 1947.
2. Copeland, D. H., and Engel, R. W. Eye Tumors in Female Rats Produced by Feeding 2-Acetylaminofluorene. Cancer Research, **10**:211, 1950.
3. Harned, B. K.; Cunningham, R. W.; and Gill, E. R. A Metabolism Cage for Small Animals. Science, **109**:489–90, 1949.
4. Heidelberger, C., and Jones, H. B. The Distribution of Radioactivity in the Mouse Following Administration of Dibenzanthracene Labeled in the 9 and 10 Positions with Carbon 14. Cancer, **1**:252–60, 1948.
5. Lindenbaum, A.; Schubert, J.; and Armstrong, W. D. Rapid Wet-Combustion Method for Carbon Determination with Particular Reference to Isotopic Carbon. Anal. Chem., **20**:1120–21, 1948.
6. Morris, H. P.; Dunn, T. B.; and Dubnik, C. S. Two Transplantable Rat Carcinomas Induced with N-Acetyl-2-Aminofluorene. J. Nat. Cancer Inst., **9**:225–28, 1948.
7. Morris, H. P., and Westfall, B. B. Distribution of N-Acetyl-2-Aminofluorene in the Rat Following a Single Feeding. J. Nat. Cancer Inst., **9**:149–54, 1948.
8. Neish, W. J. P. The Chemistry of 2-Aminofluorene. I. Reaction of 2-Aminofluorene with Pyruvic Acid. Rec. trav. chim., **67**:349–56, 1948.
9. Ray, F. E., and Geiser, C. R. 2-Acetylamino-9-C^{14}-Fluorene. Science, **109**:200, 1949.
10. ———. Synthesis of 2-Acetylaminofluorene-9-C^{14}, and 2-Acetylaminofluorene-ω-C^{14}. Cancer Research, **10**:621–24, 1950.
11. Van Slyke, D. D.; Folch, J.; and Plazin, J. Manometric Carbon Determination. J. Biol. Chem., **136**:509–41, 1940.
12. Wilson, R. H.; DeEds, F.; and Cox, A. J., Jr. The Toxicity and Carcinogenic Activity of 2-Acetylaminofluorene. Cancer Research, **1**:595–608, 1941.

A Transplantable Nephroblastoma (Wilms' Tumor) and Other Spontaneous Tumors in a Colony of Rats

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INTRODUCTION

One of the most distinctive and widespread types of the so-called "mixed tumors" is the renal neoplasm, first reported by Wilms in 1899 (10), and perhaps best described as a nephroblastoma. This tumor is found in chickens (3, 4), swine (3), cattle (3), rabbits (1, 8, 9), and rats (2), as well as in man. In spite of many attempts to transplant this tumor (2), no report of a successful transfer is available. The tumor to be described was transplanted in series.

METHODS AND RESULTS

The nephroblastoma was one of ten clearly defined neoplasms found, with a number of non-neoplastic hyperplasias, among about 500 rats examined at autopsy. It will be seen from Tables 1 and 2 that neither the ingestion of silver by about three-fifths of the rats (5–7) nor the consanguinity induced by brother-sister mating of a family of the Sherman-Mendel strain of white rat for eighteen filial generations appreciably affected the incidence of neoplasms.

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Rat A (Table 1), in which the nephroblastoma developed, was a member of the eighteenth filial generation of the inbred family and received 1:1,000 silver nitrate solution by mouth before and after nephrectomy, instead of water. The left kidney was removed when the rat was 2 months old; one kidney was removed with or without reduction of the other one in sixteen additional rats, but no tumor developed in them. The rat was well for 70 days after the operation, at which time the weight had decreased from 246 to 210 gm., and the animal became listless and was killed.

Microscopically, the tumor abutted on the renal

TABLE 1

INCIDENCE OF TUMORS

Sex and age (months)	No. of autopsies	No. of tumors	Tumors in rat (no.)	Type of Tumor
Male rats:				
Under 6	73	1	A*†	Nephroblastoma
7–12	57	0		
13–18	71	1	F*†	Fibrosarcoma of pelvis
19–24	46	2	G*†	Myxoma near salivary gland
			H*	Sarcoma of lung
25–30	17	0		
Female rats:				
Under 6	52	0		
7–12	42	1	J†	Fibrosarcoma of leg
13–18	76	3	K†	Myosarcoma of uterus
			L*	Malignant lymphoma in retroperitoneal tissues, liver and lung
			M*†	Myelogenous leukemia
19–24	62	2	N*†	Lymphangioma of neck
25–30	10	0	P	Angioendothelioma under skin of neck

* Animals given dilute solutions of silver salts instead of drinking water.
† Animals of closely related family group.

TABLE 2

PERCENTAGE INCIDENCE OF TUMORS

Groups of rats	Total no. autopsies	Total no. tumors	Percentage of tumors
Males	264	4	1.5
Females	242	6	2.5
Rats receiving silver	306	7	2.3
Rats receiving water	200	3	1.5
Rats of inbred family	307	7	2.3
Other rats	199	3	1.5
Total Animals	506	10	2.0

pelvis at one point. In other places there was a smooth line of division between the tumor and the normal renal parenchyma, with a thin but definite fibrous capsule between them. The opposite edge of the mass was very poorly defined, and many nests of characteristic tumor cells invaded the surrounding tissue. This surrounding area was composed of areolar and fibrous tissue in which were areas of edema, hemorrhage, and necrosis. The tumor (Fig. 1) was arranged in moderate-sized to very large nests; these were separated from one another by strands of normal-appearing fibrous tissue, which were often invaded by individual anaplastic cells representing the sarcoma-like elements of the tumor to be described. The sheets of tumor cells (Fig. 2) were largely composed of cells of ovoid to spindle shape, with pale cytoplasm and vesicular nuclei in which there were no conspicuous masses of chromatin. Mitoses were present in small numbers. In addition to the sarcomatous cells, there were numerous tubular structures (Fig. 3). These hollow areas were lined by cells in regular palisade arrangement. Except for this arrangement, these cells were very similar in appearance to the main cellular component of the tumor. Some of the tubular structures had a slight resemblance to primitive glomeruli.

A portion of the tumor from rat A was ground with a pestle and mortar. About 3 cc. of the resulting cloudy suspension was injected into the subcutaneous tissue and into the peritoneum of rat B, a male litter-mate of A, who had never received silver solution. A subcutaneous mass was first recognized 37 days after inoculation, and the rat was killed 95 days after inoculation, when apparently in good health except for the mass. At autopsy, many nodules of tumor tissue in the peritoneum were observed, some as much as 3 cm. in diameter. Some of them were adherent to the liver.

Microscopically (Fig. 4), the tumor again consisted of large sheets that invaded edematous areolar tissue which contained lymphocytes in moderate numbers. None of the tubular structures described in rat A were noted in Rat B. The sarcoma-like cells of this transplanted tumor seemed in some places to be slightly longer and thinner than those of the original tumor, but they were in other respects indistinguishable from them.

Material from the tumor in rat B was ground, strained through gauze, and inoculated into three rats—C, D, and E.

Rat C was a male litter-mate of rats A and B, who had never received silver solution; at 7 months of age this rat was inoculated with 1 cc. of tumor suspension into the peritoneum and 0.5 cc. into the thigh. The abdomen enlarged greatly, and the animal became lethargic and was killed 45 days after the injection. There were many light gray to red pedunculated masses up to 2 cm. in diameter in the peritoneum, and microscopic foci of invasion of the liver and spleen. The tumor (Fig. 5) was identical with that in rat B. The site of intramuscular injection in the thigh showed no tumor on gross or microscopic study, but there was some necrosis and calcification. No attempt was made to transmit this tumor of the second transplanted generation further.

Two other rats of an entirely unrelated stock were inoculated with tumor material from rat B at the time that rat C was injected. Rat D, a female about 5 months old, was injected intraperitoneally with 1 cc. of tumor suspension. There was no gain of weight or abdominal swelling. When this rat was killed 67 days later, the peritoneum was shiny and there was no evidence of tumor.

Tumor material from rat B was injected into the thigh muscles of rat E, a female about 5 months old. There was no sign of tumor at autopsy 67 days later.

COMMENT

The cells of the tubular and sarcoma-like areas of the original tumor appeared identical, except for their different arrangement. In the transplanted and retransplanted tumor, the cells were found only in sarcomatous arrangement. It is postulated that the whole complex tumor arose from one type of cell and is "mixed" in the morphological, but not in the embryological, sense.

It appears significant that this tumor was transplantable only to closely related animals.

SUMMARY

A spontaneous nephroblastoma with sarcomatous and tubular elements arose adjacent to the remaining kidney of a rat from which one kidney had been removed. The tumor was transplanted to a second, and retransplanted to a third, closely related rat. In both cases, the cells of the transmitted tumor were uniformly sarcomatous. The tumor could not be transplanted to unrelated rats. Nine other spontaneous tumors are cited.

REFERENCES

1. Bell, E. T., and Henrici, A. T. Renal Tumors in the Rabbit. J. Cancer Research, **1**:157–67, 1916.
2. Bullock, F. D., and Curtis, M. R. Spontaneous Tumors of Rats. J. Cancer Research, **14**:1–115, 1930.
3. Feldman, W. H. Neoplasms of Domestic Animals, pp. 357–72. Philadelphia and London: W. B. Saunders, 1932.
4. McKenney, F. D. Embryonal Nephroma in the Chicken. Report of Two Cases. Am. J. Cancer, **15**:122–28, 1931.
5. Olcott, C. T. Experimental Argyrosis. III. Pigmentation

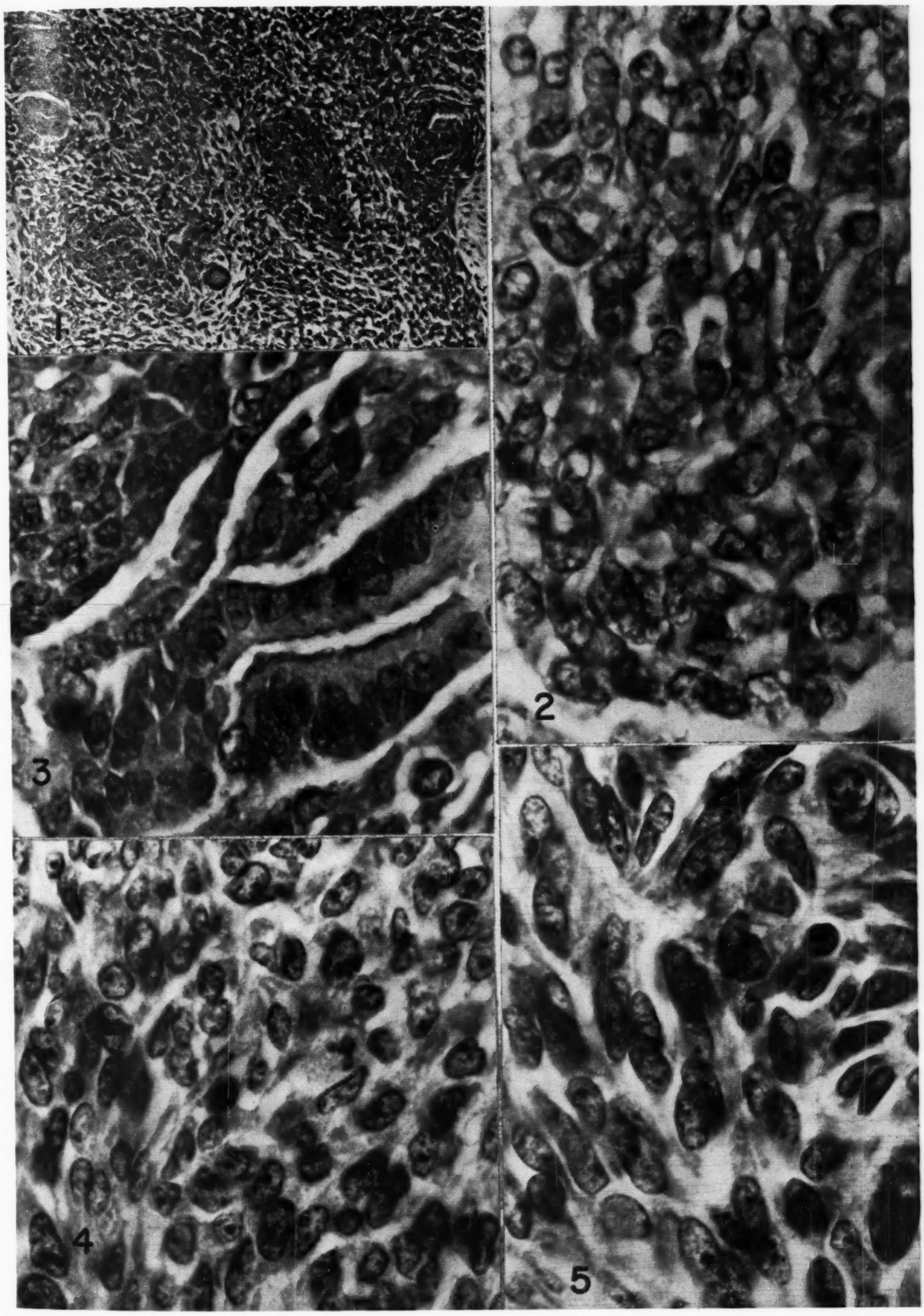

FIG. 1.—Original nephroblastoma in rat A showing adjacent tubular and sarcoma-like structure. × 225.

FIG. 2.—Detail of sarcoma-like area of tumor in rat A. × 1,080.

FIG. 3.—Detail of tubular area from rat A. × 1,080.

FIG. 4.—Sarcoma-like area from first transplanted tumor in rat B. × 1,080.

FIG. 5.—Sarcoma-like area from second transplanted tumor in rat C. × 1,080.

of the Eyes of Rats Following Ingestion of Silver during Long Periods of Time. Am. J. Path., **23**:783–91, 1947.

6. ———. Experimental Argyrosis. IV. Morphological Changes in the Experimental Animal. *Ibid.*, **24**:813–33, 1948.
7. ———. Experimental Argyrosis. V. Hypertrophy of the Left Ventricle of the Heart in Rats Ingesting Silver Salts. Arch. Path., **49**:138–49, 1950.
8. Polson, C. J. Tumours in the Rabbit. J. Path. & Bact., **30**:603–14, 1927.
9. Scott, E. Tumors of the Kidney in Rabbits. J. Cancer Research, **2**:367–71, 1917.
10. Wilms, M. Die Mischgeschwülste, **1**:1–90. Leipzig: Arthur Georgi, 1899.

Beryllium and Growth

I. Beryllium-induced Osteogenic Sarcomata*

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The pathological changes in animals resulting from the administration of compounds of the element beryllium are receiving increased attention because of the widespread use of the metal in industry and the resultant pulmonary and dermatological diseases in industrial workers.

Great impetus was given to the biochemical study of this metal by the important discovery in 1946 by Dr. Leroy Gardner that the intravenous administration of certain beryllium-containing powders to the rabbit led to the development of osteogenic sarcomas (3). The untimely death of Dr. Gardner prevented publication of the extensive data from this work. Since the time of his original observations, the production of these tumors has been confirmed in this laboratory, and it is felt that a brief account of the findings is warranted to emphasize the importance of beryllium in the field of cancer research.

METHODS

The following compounds were used for injection after being finely ground in a ball mill for 8 hours. These powders are virtually insoluble in water.

1. Beryllium phosphate. A 0.1 per cent suspension in saline was administered to five rabbits.

2. "Zinc-beryllium-silicate," a fluorescent powder containing the oxides of zinc (60 per cent), silicon (30 per cent), manganese (2 per cent), and beryllium (2.3 per cent), as well as traces of other metal oxides (SE-10). Six rabbits received this powder as a 1 per cent suspension in saline.[1]

3. A similar material containing 14 per cent beryllium oxide and 48 per cent zinc oxide (SE-6-21). This was given to four animals as a 1 per cent suspension in saline.[1]

4. Beryllium oxide. Four animals received C.P. beryllium oxide (Eimer and Amend), and five received a beryllium oxide preparation used by Dr. Gardner in his experiments, both as 1 per cent suspensions in saline.[1]

Twenty-four rabbits of unselected strains, 13 females and 11 males, with an average initial weight of $5\frac{1}{2}$ pounds, were used as experimental animals. They were kept in wire cages and fed Purina Rabbit Meal and lettuce.

The powders were injected under sterile precautions into ear veins, in doses of 5 cc. at 1-day or at 4-day intervals, in an attempt to administer a total of 1 gm. of powder.

RESULTS

Seven of the 24 rabbits developed osteogenic sarcomas, and one (which received 100 mg. of beryllium phosphate) is living and well $2\frac{1}{2}$ years after injection. Of the sixteen remaining rabbits, eight died within 3 months of the start of injections, of a multiplicity of causes not related directly to beryllium; and eight died from 14 to 28 months after injections. Most of this latter group had extensive fungus disease of the ears, complicated by pneumonia—the usual cause of death; and seven that had received beryllium oxide had fibrotic changes in the liver, a lesion which is described below. The relation of this lesion to mortality cannot be evaluated from the data.

The pertinent data on the seven animals which developed tumors are presented in Table 1.

Description of disease and pathological findings.—There was no apparent correlation between the type and the amount of material injected, the sex of the animal, and the degree of malignancy of the tumor. The data suggest that, in this study, beryllium oxide was less effective in producing tumors than the zinc-beryllium silicate. The failure of

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[1] We wish to thank the Saranac Laboratories for generously supplying these materials.

beryllium phosphate to induce tumors was probably due to the small quantity used.

The earliest signs of the disease were usually a precipitous drop in body weight, accompanied by marked crippling and by trophic changes in the skin of the affected extremity. Among the animals which died spontaneously or which were killed when obviously near death, the duration of symptoms and positive x-ray findings was from 1 to 2 months. X-rays were taken during the injection period and 6 months later, and after that as indicated by evidence of weight loss or other signs of sarcoma. The x-rays clearly demonstrated the inception and progression of the lesions (Figs. 1, 2, and 3).

TABLE 1

SUMMARY OF PERTINENT DATA ON THE SEVEN TUMOR-BEARING RABBITS

Rabbit (No.)	Compound	Amount of compound (mg.)	Injection period (weeks)	Latent period (months)	Site of primary tumor	Metastases
5	$ZnBeSiO_4$ (2.3 per cent BeO)	850	8	13	Femur	Lungs, skull, and ribs
6	$ZnBeSiO_4$ (2.3 per cent BeO)	450	8	24	Femur	None discovered
7	$ZnBeSiO_4$ (2.3 per cent BeO)	600	9	15	Humerus	Lungs, ribs, liver, kidney, skin, and omentum
11	$ZnBeSiO_4$ (14 per cent BeO)	200	0.5	11	Tibia	Lungs, liver, kidney, and omentum
21	$ZnBeSiO_4$ (14 per cent BeO)	250	2	21	Femur	Lungs, liver, kidney, and omentum
22	$ZnBeSiO_4$ (14 per cent BeO)	230	2	17	Femur	None discovered
98	BeO	1,000	16	20	Ischium	A few tiny nodules in liver and lungs

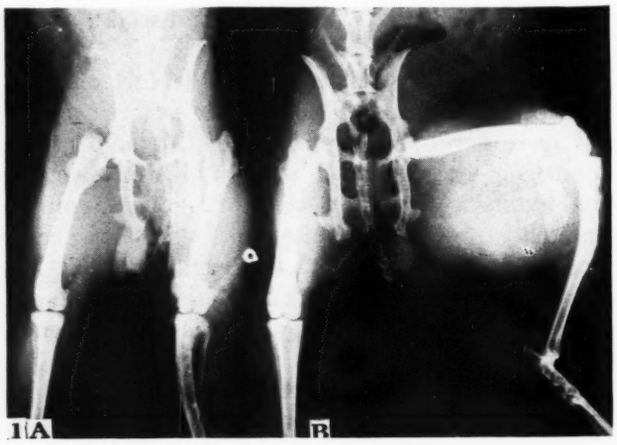


FIG. 1.—Rabbit No. 5. *A*. X-ray 18 months after injections. An early sarcoma may be seen on the medial condyle of the left femur; *B*. 1 month later the tumor has grown to immense proportions, and extensive metastases were visible in the lungs at this stage.

In two instances (rabbits Nos. 22 and 7), there was evidence of localized increase in long-bone density and even obliteration of the marrow cavity in regions other than the site of origin of the tumor. This phenomenon may be seen in the upper end of the humerus of rabbit No. 7, on the side opposite the tumor (Fig. 2). This change was not noted in the group which did not develop sarcomas, nor was there any evidence that it represented a premalignant change in the sarcoma group.

At death, the rabbits were always extremely emaciated (sometimes weighing only about one-half their maximum weight). The primary tumors were obvious on superficial examination and had usually completely compromised the function of the extremity. The cut surface of the tumors was osseous in some cases (No. 98), whereas in others it showed no gross evidence of calcification (No. 21). There were all gradations between these extremes. Areas of necrosis and hemorrhage were frequent. Note the extensive involvement shown in Figure 4.

Microscopically, these tumors were highly invasive in appearance, and the shaft of the bone at the site of origin was sometimes completely destroyed. Very little cellular or fibrous reaction to the tumor was noted. The cellular picture was that of osteogenic sarcoma, varying from extreme anaplasia to well differentiated, bone-forming tumor. Anisonucleosis, nuclear hyperchromatism, increased mitotic activity, accumulations of multinucleated giant cells, and some evidence of bone matrix formation were present in almost all sections. The accompanying figures (5, 6, and 7) are self-descriptive. Metastatic areas were generally less differentiated than the primary tumor.

The livers from all animals which had received beryllium oxide, and to a lesser extent from the silicate-injected animals, showed a fine white mottling throughout which coalesced at the liver edges and in the septa into bright white contracted areas

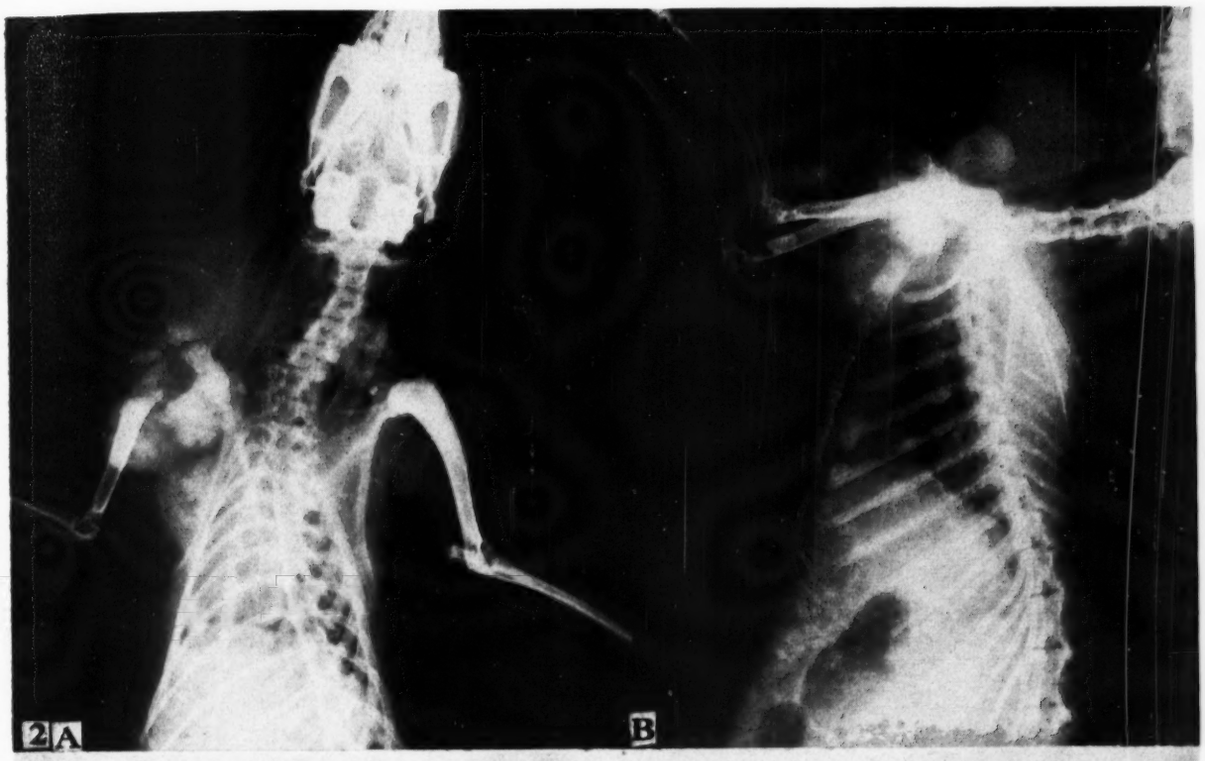


FIG. 2.—Rabbit No. 7. *A*. X-ray 15 months after the injection of beryllium—note the extensive destruction of the humerus by the sarcoma and the increased density of the bone of the upper half of the opposite humerus; *B*. numerous metastases are evident in the lungs, liver, and the peritoneal cavity.

FIG. 3.—Rabbit No. 98. *A*. Early sarcoma appearing on the ischial tuberosity 20 months after the injections; *B*. 1 month later the tumor has reached a large size. Very early metastases were found at autopsy.

(Fig. 8). Microscopically, accumulations of portal fibrous tissue, proliferation of bile duct epithelium, and collections of amorphous particulate material (which appeared to be the injected powder) were evident (Fig. 9). Similar accumulations of particles were found in the spleen. These changes were as commonly found in those animals which failed to develop tumors as in those which developed tumors.

One rabbit (No. 95), which received beryllium oxide but did not develop a tumor, had a small spleen, the Malpighian corpuscles of which were

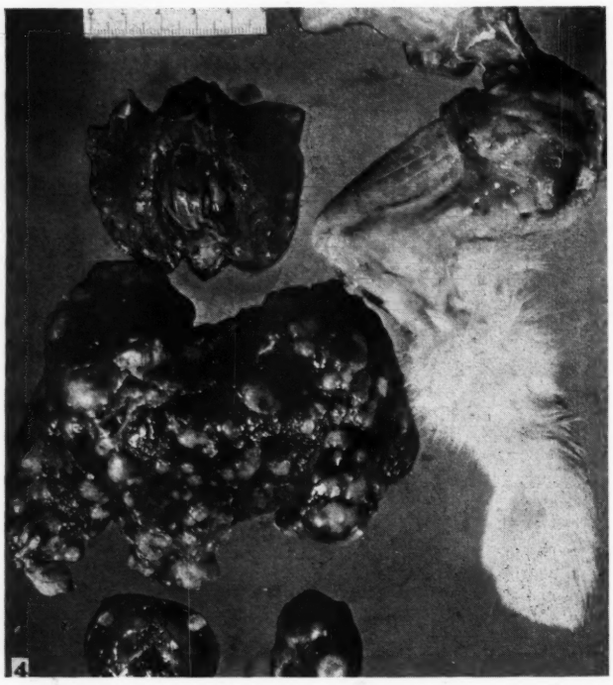


FIG. 4.—Rabbit No. 7. Lungs, liver, kidneys, and the primary tumor removed to show the massive involvement characteristic of these tumors. This photograph was taken approximately 6 weeks after the first appearance of symptoms.

filled with large, multinucleated giant cells. These cells were loaded with particles (Fig. 10).

Transplantation —Several attempts were made to transplant the sarcoma tissue. Using aseptic precautions, fresh tissue was cut into small pieces in saline and implanted in the following sites: tumor tissue from one rabbit was introduced by trocar directly into the distal femoral marrow cavity of two normal rabbits; tissue from two other tumors was introduced into the anterior chamber of the eyes of four guinea pigs and also under the skin of two rabbits; finally, tissue from another rabbit was implanted into the anterior chamber of a normal rabbit's eyes. All these transplanted tissues failed to grow.

Alkaline phosphatase studies.—Alkaline phosphatase determinations on tissues and serum were done in a manner similar to that described by Franseen and McLean (2), with minor modifications.[2] Studies on activation and inhibition of the enzyme were performed as described in a previous paper (4). A selection of relevant data on tumor-bearing rabbits is presented in Table 2.

The range of serum alkaline phosphatase activity in six rabbits (four rabbits which never developed tumors and two rabbits in the pre-tumor stage) was 0.4–2.1 units per cubic centimeter of serum. In the two animals in which phosphatase determinations were done when the sarcoma was just visible by x-ray (Nos. 6 and 7), the values were slightly but significantly elevated. Thereafter, there was a rapid and dramatic rise in activity, which seemed to parallel the extent of metastases rather than the size of the primary lesion. There was no rise in serum phosphatase in animals with no tumor.

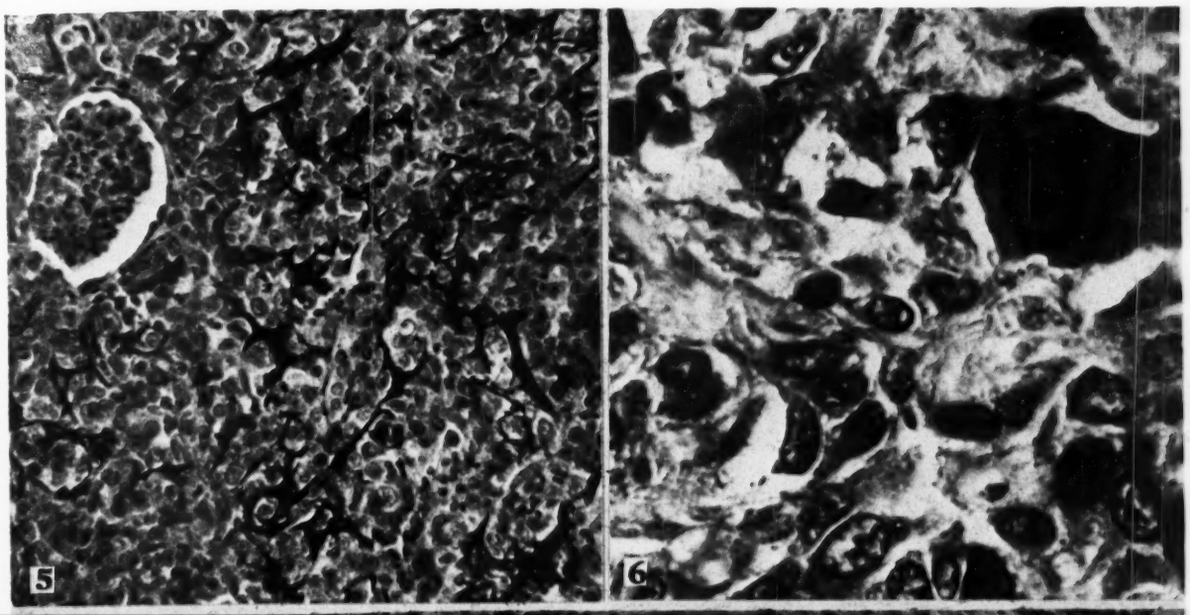
Tissue alkaline phosphatase determinations revealed a high activity in the tumor tissue in every instance (Table 2). The areas of dense bone described earlier and of bone adjacent to the tumor showed significant elevations in activity.

Because of the known inhibitory effect of beryllium upon the activity of alkaline phosphatase (1, 4, 5), a study of the effect of beryllium upon the phosphatase of this particular tumor was undertaken. The results of this study clearly showed that the glycerophosphatase of dialyzed Be-sarcoma extracts was activated by magnesium and inhibited by beryllium in a manner identical with that described for other tissues (4) over a pH range of from 7 to 10.

DISCUSSION

The production of an extremely malignant tumor with a simple metallic oxide is of fundamental importance in the field of cancer research. That beryllium itself is a true sarcomagenic agent is strongly suggested by the fact that, as shown by Gardner (3), beryllium phosphate, as well as the oxide and silicate, is an effective tumor inducer. It is not known whether a local physical irritation factor contributed by an insoluble powder is an adjuvant to the chemical action of ionic beryllium. Soluble salts of beryllium have not produced tumors, but to do this would involve long-term injections of beryllium in small doses, a tedious procedure which has not been undertaken. Beryllium compounds have been injected into rabbits, rats, and guinea pigs, and of these the rabbit is apparently the only susceptible species. Elucida-

[2] D. M. Tibbetts, unpublished material.



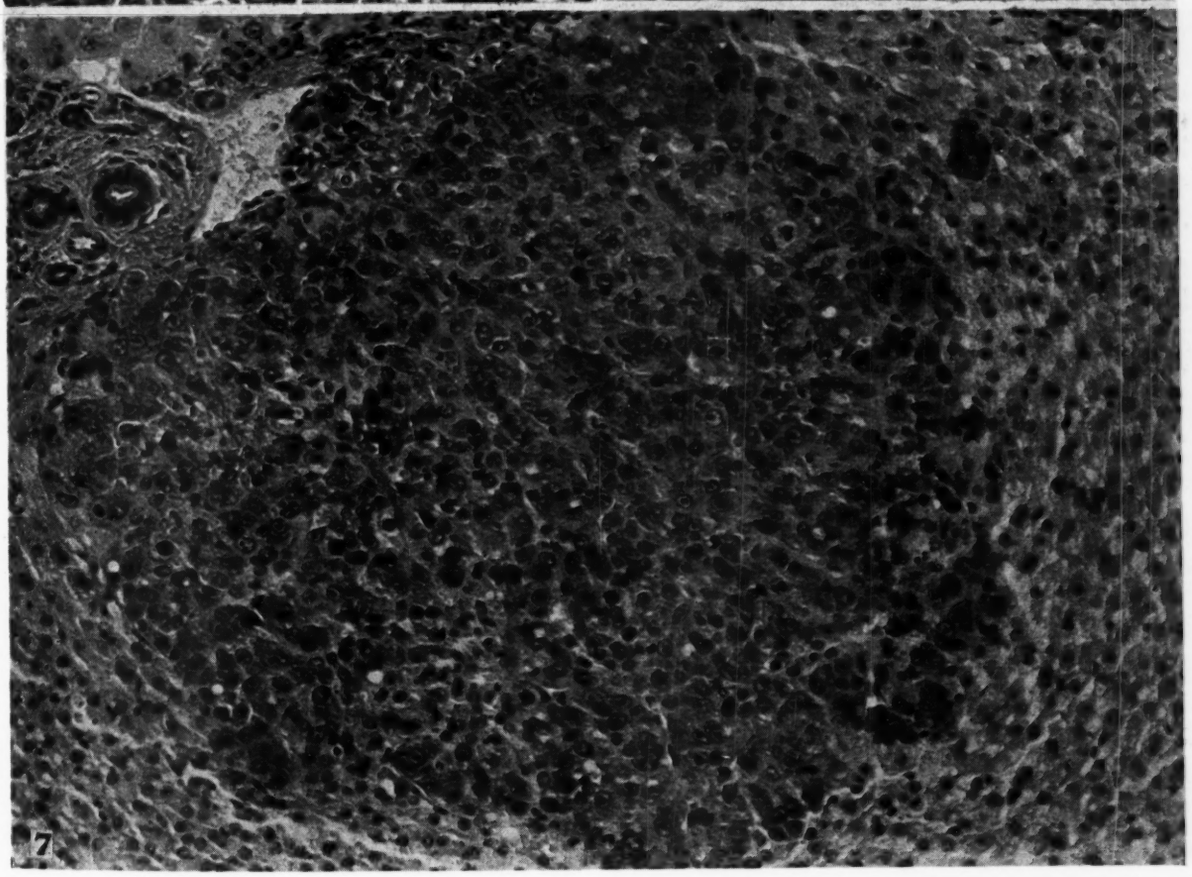


FIG. 5.—Rabbit No. 7. A section of a kidney metastasis showing tumor tissue invading the kidney cortex and laying down some bone matrix. × 100.

FIG. 6.—Rabbit No. 21. Metastatic area in the liver illustrating a high degree of anaplasia. Note the giant cell, anisonucleosis, and hyperchromatism. × 600.

FIG. 7.—Rabbit No. 98. A miliary metastatic nodule in the liver. This was one of the few metastases found in this animal and was derived from a primary lesion which was more differentiated than most of the other tumors. × 100.

FIG. 8.—Rabbit No. 20. Gross specimen of the fresh liver showing the dense white contracted areas of fibrous tissue, in the meshes of which were found what appeared to be deposits of injected powder.

FIG. 9.—Rabbit No. 98. Section of the liver depicting the early fibrotic changes and what appear to be beryllium oxide deposits in the portal area. This type of lesion apparently eventuates in the picture seen in Figure 8. × 100.

tion of the metabolic differences between the rabbit and other mammals might shed light on the mechanism of the response.

The only well defined biochemical action of beryllium known at this time is its inhibition of alkaline phosphatase *in vitro*. This phenomenon occurs with concentrations of beryllium low enough (10^{-7} M) to suggest its possible importance *in vivo*. The fact that phosphatase in sarcoma

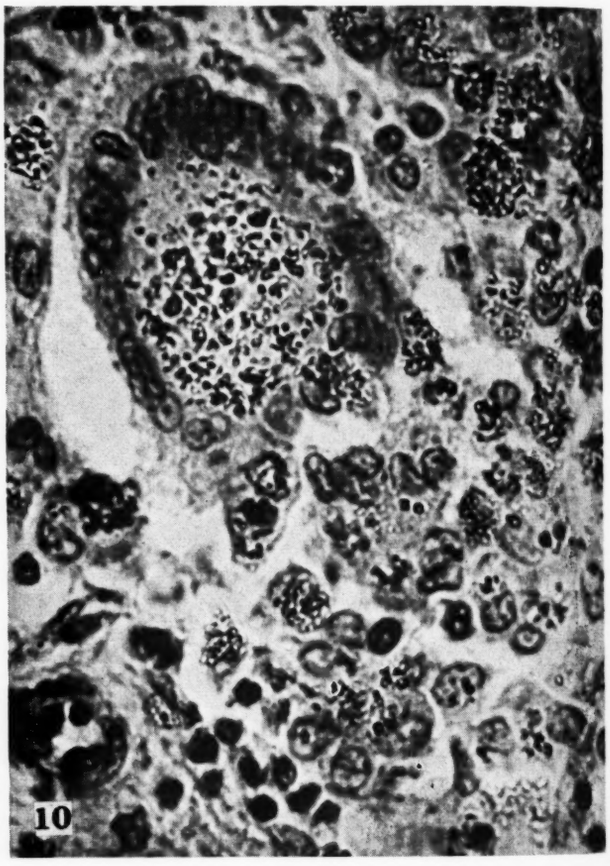


FIG. 10.—Rabbit No. 95. Section through the Malpighian corpuscle of the spleen showing scattered foci of particulate material, and a large giant cell containing much of the powder. × 603.

is inhibited by beryllium in a manner similar to normal bone phosphatase suggests that this enzyme is not altered in its properties. However, our criteria for characterizing phosphatases are so limited that it is impossible to say at this time whether or not the enzyme is different. The finding does not exclude the possibility, however, that the action of beryllium upon normal bone phosphatase may have been initially important in tumor induction. Elucidation of the mechanism involved here must await clarification of the significance of phosphatases in general. Evidence is accumulating that beryllium has no similar inhibitory effect upon a wide variety of other essential magnesium-

TABLE 2

SERUM AND TISSUE ALKALINE PHOSPHATASE ACTIVITY IN TUMOR-BEARING RABBITS (UNITS*)

	Serum			Tissue				
							Metastatic site	
Rabbit (No.)	During injection period	At appearance of tumor	At death	Normal	Bone Dense or near tumor	Tumor	Normal	Metastasis
5		5.1	12.6	1.0	2.4	130.0	1.5 (liver)	16.4 (lung)
6	0.9		0.5					
7		2.9	201.3	2.3		66.1		33.3 (lung) 99.5 (liver) 108.0 (kidney)
11				0.6	12.9	254.0	2.5 (liver) 8.0 (kidney)	179.3 (liver)
21			8.4					
22				1.0	8.6	92.6		
98	1.5–0.4		5.9	1.0		23.7		

* Milligrams P split per 100 cubic centimeters of serum or 1 gm. of tissue in 1 hour at 37° C. and pH 9.0.

activated enzymes, and there is a growing respect for the importance of phosphatases in protein metabolism and the growth processes. One might assume that the phosphatases control the level of organic phosphates which store energy for synthesis and that any agent which selectively inhibits the hydrolytic action of these enzymes might be expected to increase synthetic activity. However, evidence is now appearing (6) that alkaline phosphatase has the ability to transfer phosphate from one organic molecule to another, an effect which might have the opposite significance in synthesis.

It is the purpose of the present investigations in these laboratories to correlate such specific biochemical actions as these with a variety of growth responses to this metal.

SUMMARY

1. Twenty-four rabbits were injected with insoluble beryllium compounds, and seven animals developed osteogenic sarcomas in from 11 to 24 months after injection. These tumors were highly malignant and metastasized rapidly. Seven other injected rabbits—apparently dying of unrelated causes—were found to have remarkably fibrotic and contracted livers.

2. All attempts to transplant these tumors met with failure.

3. The serum alkaline phosphatase activity rose rapidly, paralleling the spread of the tumor throughout the body, and the activity of this enzyme in the tumor tissue itself was extremely high. Magnesium was found to activate, and beryllium to inhibit, the phosphatase associated with the sarcoma in the same manner that these metals affect normal alkaline phosphatase.

4. The importance of an extremely malignant sarcoma produced by a simple metal oxide, in the light of the known effects of beryllium on certain enzyme systems, is discussed.

ACKNOWLEDGMENTS

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REFERENCES

1. DUBOIS, K. P.; COCHRAN, K. W.; and MAZUR, M. Inhibition of Phosphatases by Beryllium and Antagonism of the Inhibition by Manganese. Science, **110**:420–22, 1949.
2. FRANSEEN, C. C., and MCLEAN, R. The Phosphatase Activity of Tissues and Plasma in Tumors of Bone. Am. J. Cancer, **24**:299–317, 1935.
3. GARDNER, L. U. Osteo-Sarcoma from Intravenous Beryllium Compounds in Rabbits. Fed. Proc., **5**:221, 1946.
4. GRIER, R. S.; HOOD, M. B.; and HOAGLAND, M. B. Observations on the Effects of Beryllium on Alkaline Phosphatase. J. Biol. Chem., **180**:289–98, 1949.
5. KLEMPERER, F. W.; MILLER, J. M.; and HILL, C. J. The Inhibition of Alkaline Phosphatase by Beryllium. J. Biol. Chem., **180**:281–88, 1949.
6. MEYERHOF, O., and GREEN, H. Transphorylation by Alkaline Phosphatase in the Absence of Nucleotides. II. J. Biol. Chem., **183**:377–90, 1950.

Citric Acid Metabolism in Carcinogenesis and Its Relationships to Calcium Metabolism*

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Previous studies on the calcium metabolism of epidermis undergoing carcinogenesis (2, 10, 11) have suggested the possibility that the action of one or more calcium-binding substances may account for the decreased calcium content in hyperplastic epidermis and in a squamous-cell carcinoma. The present investigation has been devoted to a study, in normal and cancerous tissues of the mouse, of the content of citric acid, a substance known to form slightly dissociated complexes with calcium (7).

Several tumor tissues were analyzed by Dickens (5), who concluded that tumor tissue contains more citric acid than most normal tissues, with the exception of skin, bone, hair, and the tissues comprising the seminal vesicle. A high content of citric acid was found in the Walker 256 tumor of the rat by Haven, Randall, and Bloor (8). The latter investigators also reported a markedly higher content of citric acid in the necrotic center of this tumor than in the viable periphery. These findings differ from those of Dickens (5), who found a higher citrate level in the non-necrotic portions of the Crocker sarcoma.

MATERIALS AND METHODS

The following tissues of the mouse were analyzed for their citric acid contents: normal and hyperplastic Swiss strain epidermis, a Swiss strain transplanted squamous-cell carcinoma, C strain liver and transplanted hepatoma,[1] Leaden strain liver and transplanted hepatoma,[2] and C3H strain muscle and transplanted rhabdomyosarcoma.[3]

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[1] Obtained from Dr. H. B. Andervont of the National Cancer Institute.

[2] Obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine.

[3] Otained from Dr. E. U. Green, Institute for Cancer Research and the Lankenau Hospital Research Institute, Philadelphia.

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Technics for shaving the mice, applying methylcholanthrene, and separating the epidermis from the dermis have been previously described (1, 3). Thigh, gluteal, and lower back muscles were used for normal muscle analyses on C3H strain mice. Subcutaneous transplants of the various tumors were removed when the neoplasms attained a diameter of between 0.5 and 1 cm. (with the exception of several C strain hepatomas, which were larger). Fresh tissue was removed, weighed on a Roller-Smith precision balance, and ground in a mortar with about 5 ml. of 10 per cent trichloroacetic acid, with the aid of purified sand when necessary. The contents of the mortar were transferred quantitatively to a 40-ml. centrifuge tube by rinsing both the mortar and pestle several times with 10 per cent trichloroacetic acid and by adding the washings to the centrifuge tube.

The citric acid content of the tissues was determined by the method of Taussky and Shorr (13) and Taussky (12). The color reaction used, however, was that of Natelson, Pincus, and Lugovoy (9). A number of modifications of these procedures were necessitated by the fact that they were originally devised for blood and urine analyses. The entire procedure will, therefore, be outlined, in order to clarify these modifications.

The contents of the centrifuge tube were stirred well, allowed to stand at room temperature for about 10 minutes, and then centrifuged for the same period of time at 1,500 r.p.m. The supernatant was then filtered through a 7-cm. Whatman No. 42 filter paper into a graduated cylinder and the volume of the filtrate recorded. Two ml. of the filtrate was pipetted into a 60-ml. glass-stoppered Pyrex bottle to which 9 ml. of distilled water was added to bring the sample to volume. In order to determine recoveries, a known amount of citric acid was added to the tissue filtrate, and the solution then brought to volume (11 ml.). One ml. of 20 per cent trichloroacetic acid was added to all standard solutions, to maintain a constant concentration of the acid in all samples, before making a similar dilution with distilled water.

The next step was the addition of sulfuric acid. It was found (Table 1) that the addition of 3.7 ml. of 27 N sulfuric acid to epidermal samples gave the best recoveries, and 4.4 ml. of 27 N sulfuric acid also produced better results than any other concentrations of acid with liver and muscle samples. The normalities of sulfuric acid prior to the oxidation stage were 6.36 and 7.24, respectively. It may also be mentioned that, although varying the normality of sulfuric acid had some effect on the results obtained on tissue samples, no changes were noted with standard solutions when the normality of the acid was varied.

TABLE 1

RECOVERIES OF ADDED CITRIC ACID

Tissue	Sample no.	Normality of H_2SO_4	Citric acid content (γ)	Recovery sample* (γ)	Non-recovery sample† (γ)	Added citric acid‡ (γ)	Recovery (per cent)
C strain liver	1	7.24	13.1	6.0	2.8	3.57	95.4
	2	11.6	18.3	5.3	2.8	3.57	83.3
C strain hepatoma	3	7.24	174	11.3	8.2	3.57	96.0
	4	11.6	162	7.8	5.3	3.57	88.0
Leaden strain liver	5	7.24	27.0	8.5	4.3	3.57	108
	6A	1.08	29.7	9.2	4.3	3.57	117
	6B	1.84	39.4	10.2	5.7	3.57	110
	6C	3.86	24.9	8.8	3.6	3.57	123
	6D	11.6	19.4	5.3	2.8	3.57	83.3
Leaden strain hepatoma	7	7.24	69.9	5.7	2.8	3.57	89.6
	8	11.6	52.2		2.5		
C3H strain muscle	9	7.24	24.5	5.3	1.4	3.57	107
	10A	6.10	25.4	5.7	1.1	3.57	122
	10B	6.36	16.2	4.3	0.7	3.57	101
	10C	6.63	32.4	4.3	1.4	3.57	86.6
	10D	7.37	25.4	3.6	1.1	3.57	77.2
	10E	11.6	32.4	4.3	1.4	4.28	77.4
C3H strain rhabdomyosarcoma	11	7.24	47.8	5.3	2.1	3.57	93.5
Swiss strain epidermis	12A	3.86	272	12.0	7.8	3.57	106
	12B	5.40	247	11.6	7.1	3.57	109
	12C	6.75	247	10.2	7.1	3.57	95.8
	12D	11.6	209	6.7	6.0	1.43	90.2
3 paintings, methylcholanthrene	13	6.36	196	11.6	7.8	3.57	102
6 paintings, benzene	14	6.36	529	7.8	3.9	5.00	87.8
12 paintings, benzene	15A	5.96	478	12.4	9.6	3.57	94.3
	15B	6.36	478	13.0	9.6	3.57	98.8
	15C	6.63	478	11.6	9.6	3.57	88.2
	15D	7.00	478	12.7	9.6	3.57	96.6
24 paintings, methylcholanthrene	16	6.36	206	10.6	6.4	3.57	106
Squamous-cell carcinoma	17	6.36	69.6	6.0	2.5	3.57	99.0

* Citric acid content of final aliquot of recovery sample.
† Citric acid content of final aliquot of nonrecovery sample.
‡ Final aliquot of amount of citric acid added to recovery sample.

After the addition of a suitable amount of sulfuric acid from a burette, the solution was shaken and placed for about 5 minutes in a water bath maintained at from 15° to 18° C. Then the following solutions were added from a burette: 0.5 ml. of 40 per cent manganese sulfate, 0.5 ml. of bromide-bromate solution (9), and 1.5 ml. of 5 per cent potassium permanganate. The mixture was shaken thoroughly and allowed to stand for about 30 minutes in the water bath maintained at from 15° to 18° C., after which time suction was applied to remove the bromine fumes. Four ml. of 40 per cent ferrous sulfate (freshly prepared) was added, and the mixture was shaken and was allowed to stand for about 3 minutes, after which time suction was again applied as an added precaution. Seven ml. of normal heptane was added from a burette, and the mixture was shaken in the Aloe shaking machine for 6 minutes. The contents were poured into a separatory funnel and washed about 5 times (to neutrality) with doubly distilled water. Upon complete separation of the two layers, 5 ml. of the heptane layer was pipetted into a 30-ml. glass-stoppered Pyrex bottle.

Three and one-half ml. of buffered thiourea solution (9) was delivered from a burette into the bottle, which was then stoppered and shaken in the

Aloe shaking machine for 6 minutes. The contents of the bottle were poured into a 12-ml. centrifuge tube and centrifuged for 5 minutes at 2,000 r.p.m. The aqueous layer was then pipetted into a 10-mm. Coleman cuvette and read at a wave length of 445 mμ in the Coleman spectrophotometer.

The formula used for the calculation of the citric acid levels of tissue samples is as follows:

$$T = \frac{F \times K \times V_1 / V_2}{W}$$

where T is the citric acid content of the tissue (γ/gm), F the citric acid content of the final aliquot (γ), K (1.40) the proportion of the original aliquot used, V_1 the volume of the original tissue filtrate (ml.), V_2 the volume of filtrate used in the analysis (ml.), and W the weight of the tissue (gm.).

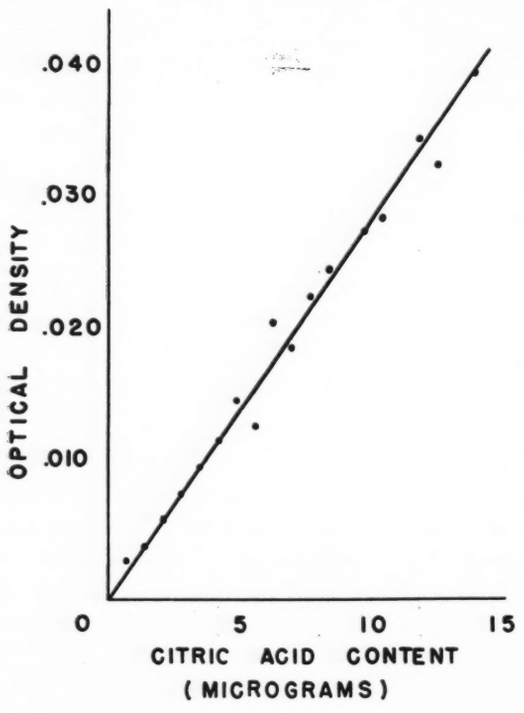

FIG. 1.—Standard curve

Twelve samples, including one blank and two standards, were run simultaneously, although this number was sometimes varied. However, one blank and at least one standard were always run with tissue samples. A satisfactory standard curve was plotted from the results of the analyses of 27 standard solutions (Fig. 1).

RESULTS

Greenstein's observation (6) that "no matter how, or from which tissues tumors arise, they more nearly resemble each other chemically than they do normal tissues or than normal tissues resemble each other" has received further support from this present work. The citric acid contents of the tumors analyzed were more alike than were the individual tumors in respect to their normal homologs. Although a great variation was shown between the various normal tissues that were analyzed, a relatively small variation was found between the citric acid contents of the tumors (Tables 2 and 3 and Fig. 2). With the exception of epidermis, a marked increase in the citric acid contents of all tumors, as compared with values for normal tissues, was consistently displayed. The citric acid content of the squamous-cell carcinoma, however, presented a very marked decrease from the normal values. Thus, all tumors analyzed tended to attain an almost identical citric acid content, irrespective of the citric acid level of their normal homologs.

TABLE 2

THE CITRIC ACID CONTENT OF LIVER AND MUSCLE TUMORS AND THEIR NORMAL HOMOLOGS (NORMALITY OF SULFURIC ACID, 7.24)

Tissue	Number of mice	Average citric acid concentration (γ/gm., wet wt.)
C strain liver	6	11.2
C strain hepatoma	4	130.5
C strain blood	about 20	29.1
Leaden strain liver	6	37.9
Leaden strain hepatoma	4	64.8
Necrotic portion of leaden strain hepatoma	1	53.1
C3H strain muscle	3	30.0
C3H strain rhabdomyosarcoma	4	68.0
Necrotic portion of rhabdomyosarcoma	2	91.6

Analyses of the necrotic portions of the Leaden strain hepatoma, squamous-cell carcinoma, and rhabdomyosarcoma indicated slight differences in the citric acid contents of these areas as compared to the viable zones (Tables 2 and 3). The slight decrease shown by the necrotic portion of the Leaden strain hepatoma is certainly not significant, while the slight increases shown by the rhabdomyosarcoma and squamous-cell carcinoma are unquestionable, since there is no overlapping of values. Certainly, however, the results of this work cannot explain the diametrically opposed findings of Dickens (5) and Haven, Randall, and Bloor (8) on the citric acid contents of necrotic tissue. One possibility, however, is that the citrate contents of necrotic areas analyzed by these investigators were affected by bacterial contamination. The tumors used in this present work were probably not contaminated, as revealed by cultures made from several of these tissues. Neglecting the possibility of contamination, it would seem

TABLE 3

CITRIC ACID IN EPIDERMAL CARCINOGENESIS
(NORMALITY OF SULFURIC ACID, 6.36)

No. of mice	No. of paintings	Citric acid content (γ/gm., wet wt.)
	Normal epidermis	
4		539
4		375
4		463
3		482
Total: 15		Average: 465
	Benzene-treated mice	
9	3	417
5	3	503
5	3	400
9	3	385
Total: 28		Average: 426
4	6	275
3	6	155
3	6	377
3	6	529
Total: 13		Average: 334
4	12	305
5	12	399
6	12	393
7	12	389
8	12	478
Total: 30		Average: 393
4	24	279
5	24	321
6	24	336
7	24	226
8	24	277
Total: 30		Average: 288
	Methylcholanthrene-treated mice	
5	3	206
4	3	242
8	3	205
7	3	196
Total: 24		Average: 212
4	6	250
4	6	163
3	6	256
3	6	195
Total: 14		Average: 216
3	12	272
5	12	218
6	12	196
7	12	203
8	12	192
Total: 29		Average: 216
3	24	352
5	24	208
6	24	240
10	24	206
Total: 24		Average: 252
	Squamous-cell carcinoma	
4		69.6
2		70.8
3		81.2
Total: 9		Average: 73.9
	Necrosis from squamous-cell carcinoma	
Total: 1		110

at present that there is no set pattern for the citrate content in necrotic portions of tumors with respect to the non-necrotic portions of the same tumors. Obviously, more work is needed to settle the issue on this particular side line.

The application of methylcholanthrene resulted in a very marked initial decrease in the epidermal citric acid content (Fig. 3). This diminution was maintained throughout hyperplasia following a number of treatments with the carcinogen. A second noteworthy decrease may also be noted between hyperplastic epidermis and the squamous-cell carcinoma.

Benzene-treated epidermis also decreased in citrate content, but not so markedly as the methylcholanthrene-treated epidermis (Table 3). No comparable decrease in benzene controls was found in epidermal calcium analyses (2, 10, 11).

DISCUSSION

The results of this present work indicate that some very definite, clear-cut changes take place in the citric acid contents of tumor tissues as com-

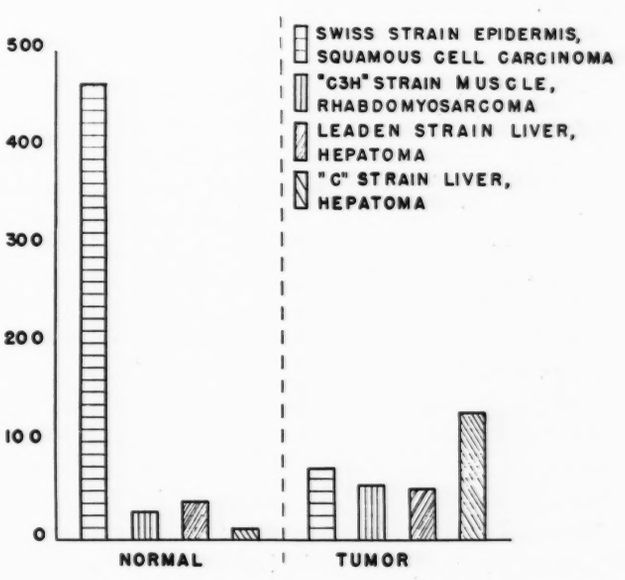

FIG. 2.—The citric acid content of various tumors and their normal homologs (γ/gm.).

pared to their normal homologs. Such changes probably reflect some metabolic alterations in the biochemistry of tumor tissue. The nature of these metabolic processes, however, is uncertain.

Body needs for citric acid are probably obtained by processes of intermediate metabolism (4). Certainly, citric acid cannot be directly derived from nutritional sources in the same manner that calcium and other minerals are obtained. Bone also appears to be an unlikely source (4), despite the high content of citrate in its substance (5). It is, therefore, most probable that the source of citric

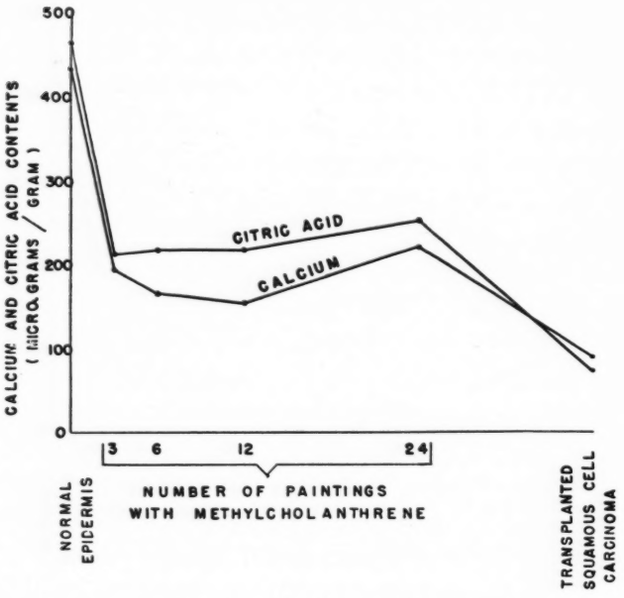

FIG. 3.—Calcium and citric acid in epidermal carcinogenesis.

acid is from the aerobic oxidation of carbohydrates, since citric acid is known to be formed as a by-product of the tricarboxylic acid cycle. Thus, it might be possible to interpret the present data on the basis of changes taking place in the aerobic oxidation of carbohydrates. Much further work is necessary in order to investigate these possibilities.

The correlations between the calcium and citric acid metabolisms of normal and hyperplastic epidermis and a transplanted squamous-cell carcinoma reveal some interesting data (Fig. 3). The changes in the epidermal contents of these two substances are similar in the carcinogenic processes, suggesting the possibility of the two compounds being bound together, probably in the form of a diffusible complex, throughout the process. It is also conceivable that calcium is removed from the site of hyperplasia to a great extent by citric acid, the two substances passing together into the tissue fluids and, hence, into the blood stream. It, therefore, seems very likely that there is some correlation between the present analyses and those of calcium (2, 10, 11).

A probable formula for calcium citrate (Ca_3Cit_2) has been presented by several investigators (7). Calculating the mole ratios between calcium and citric acid in the various epidermal tissues analyzed (Table 4) clearly indicates that, according to this or any other probable formula, only a part of the calcium present in the epidermis or squamous-cell carcinoma can theoretically be bound to citric acid. This fact, however, does not in any way destroy the possibility that citric acid plays an important role in calcium removal. Other substances, such as nucleoproteins and possibly other tricarboxylic acids, undoubtedly

TABLE 4

THE MOLE RATIOS BETWEEN CALCIUM AND CITRIC ACID IN EPIDERMAL CARCINOGENESIS (EXPRESSED AS NUMBERS OF MOLES OF CALCIUM/MOLE OF CITRIC ACID)

Number of paintings with methylcholanthrene	Moles of calcium/mole of citric acid
Normal, untreated epidermis	4.51
3 paintings	4.32
6 paintings	3.63
12 paintings	3.42
24 paintings	4.20
Squamous-cell carcinoma	5.84

share in the functioning of such a mechanism, if it exists at all.

Morphological evidence presented by Banyen[4] may necessitate changes in the interpretations of chemical data from the idea that the individual cell changes chemically in carcinogenesis to the concept that chemical changes are merely due to changes in cell types. It was shown by this investigation that basal cells remain fairly constant in hyperplasia, while granular and spinous cells increase greatly in number but always are present in about the same proportion. The interpretation of calcium and citric acid analyses in epidermal hyperlasia may therefore be modified as follows: The contents of these substances in normal epidermis are very high in the basal layer and much lower in the granular and spinous layer. In epidermal hyperplasia, an increase in the number of granular and spinous cells would serve to decrease the amounts of calcium and citric acid per unit weight of epidermis. Therefore, changes in these substances can be explained on the basis of cell ratios, and not on the basis of an alteration in the

[4] D. Banyen, Study of Cell Types in Mouse Epidermis during Methylcholanthrene Carcinogenesis (in preparation).

chemistry of the individual cell. This concept, of course, is purely hypothetical, but certainly worthy of our attention. It may be that the two conflicting hypotheses, chemical and morphological, each play a definite integrated role in the dynamics of carcinogenesis.

SUMMARY

1. A method for the determination of citric acid in tissues has been outlined.

2. It was found that each normal tissue examined had a content of citric acid characteristic for that tissue, while the tumors, regardless of derivation or source, showed very similar levels of citric acid.

3. An attempt has been made to correlate the results in terms of calcium and carbohydrate metabolism and cell ratios.

REFERENCES

1. BAUMBERGER, J. P.; SUNTZEFF, V.; and COWDRY, E. V. Methods for the Separation of Epidermis from Dermis and Some Physiologic and Chemical Properties of Isolated Epidermis. J. Nat. Cancer Inst., **2**:413–23, 1942.
2. CARRUTHERS, C., and SUNTZEFF, V. Chemical Studies on the Mode of Action of Methylcholanthrene on Mouse Epidermis. Cancer Research, **3**:744–48, 1943.
3. ———. Influence of Limited Application of Methylcholanthrene upon Epidermal Iron and Ascorbic Acid. J. Nat. Cancer Inst., **3**:217–20, 1942.
4. CLASS, R. N., and SMITH, A. H. The Skeleton as a Source of Endogenous Citric Acid. J. Biol. Chem., **151**:363–68, 1943.
5. DICKENS, F. The Citric Acid Content of Animal Tissues, with Reference to Its Occurrence in Bone and Tumour. Biochem. J., **35**:1011–23, 1941.
6. GREENSTEIN, J. P. Biochemistry of Cancer, p. 305. 1st ed. New York: Academic Press, Inc., 1947.
7. HASTINGS, A. B.; MCLEAN, F. C.; EICHELBERGER, L.; HALL, J. L.; and DACOSTA, E. The Ionization of Calcium, Magnesium and Strontium Citrates. J. Biol. Chem., **107**: 351–70, 1934.
8. HAVEN, F. L.; RANDALL, C.; and BLOOR, W. R. The Citric Acid Content of Tumor Tissue and of Tumor-bearing Rats. Cancer Research, **9**:90–92, 1949.
9. NATELSON, S.; PINCUS, J. B.; and LUGOVOY, J. K. Microestimation of Citric Acid; a New Colorimetric Reaction for Pentabromoacetone. J. Biol. Chem., **175**:745–50, 1948.
10. SUNTZEFF, V., and CARRUTHERS, C. The Effect of Methylcholanthrene upon Epidermal Sodium and Calcium. Cancer Research, **3**:431–33, 1943.
11. ———. Potassium and Calcium in Epidermal Carcinogenesis Induced by Methylcholanthrene. J. Biol. Chem., **153**:521–27, 1944.
12. TAUSSKY, H. H. A Microcolorimetric Method for the Determination of Citric Acid. II. A Note on the Substitution of Ferrous Sulfate for Hydrazine Sulfate as the Reducing Agent. J. Biol. Chem., **181**:195–98, 1949.
13. TAUSSKY, H. H., and SHORR, E. A Microcolorimetric Method for the Determination of Citric Acid. J. Biol. Chem., **169**:103–18, 1947.

The Uptake and Distribution of Radioactive Phosphorus in Chicken Eggs Containing a Rapidly Growing Mammary Tumor of a C3H Mouse*

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Tumors of rats and especially of mice have been grown in chicken eggs for a number of years. Tumors of sufficient size and uniformity as to be used in chemotherapy have been produced by the yolk sac technic, whereby the macerated tumor is injected into the yolk sac. The advantages of such a technic for screening chemicals for any tumor-inhibiting effects have already been pointed out by Taylor (1) and thus will be reviewed here only briefly.

The rapidly growing tumor and chick embryo grow within the closed system of the egg, sharing the same blood stream but without any physical contact between the two. It is thus possible to inject a substance between the egg shell and the chorio-allantoic membrane (analogous to a subcutaneous injection in mice) and, by weighing the tumor and chick embryo of both control and treated eggs, to determine whether the substance (*a*) has no effect, (*b*) inhibits tumor growth specifically, (*c*) inhibits embryo growth specifically, or (*d*) inhibits fast-growing tissues, in general.

The following experiments were undertaken to determine the manner of uptake and distribution of a substance injected between the chorio-allantoic membrane and the egg shell. For this purpose radioactive phosphorus in the form of Na_2HPO_4 was used.

METHODS

A mammary tumor of a C3H mouse was grown in chicken eggs using the yolk sac injection method (2). On the twelfth day of incubation (8 days after tumor inoculation), radioactive P^{32} in a 1 per cent aqueous solution of Na_2HPO_4 was injected between the chorio-allantoic membrane and the egg shell. The eggs were opened 48 and 96 hours later, and samples were taken of the various portions to be compared. These included the mouse tumor, the chick embryo skeleton, blood, allantoic fluid, yolk, and albumen. In another series the tumor and embryo brain and liver were compared as to P^{32} uptake.

The tissues were thoroughly macerated, and samples were dried to a constant weight which was then accurately determined. The activity of the sample was determined by use of a Geiger-Müller counter. The thickness of the samples was never enough to necessitate corrections for self-absorption. Decay corrections were made by comparison with a control sample of radioactive phosphate prepared from the same solution used for injection. The counts/minute/100 mg dry weight were used as the basis for comparison.

RESULTS

When P^{32} was injected between the egg shell and the chorio-allantoic membrane it was gradually taken up by the blood stream and distributed to the various parts of the egg. Table 1 shows the uptake and distribution of the P^{32} 48 and 96 hours after its injection.

TABLE 1

DISTRIBUTION OF P^{32} IN CHICK EMBRYO AND TUMOR

Material	Hours after P^{32} injection	Counts/minute/100 mg dry wt.
Yolk	48	72
	96	9
Albumen	48	146
	96	35
Tumor	48	769
	96	2,600
Chick embryo skeleton	48	1,000
	96	12,900
Blood	48	11,800
	96	961
Allantoic fluid	48	56,500
	96	43,900

At 48 hours after injection most of the P^{32} was taken up by the blood and had passed through the system. The largest portion of it was excreted into

* This work was done at the University of Texas under a Rosalie B. Hite Cancer Fellowship.

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the allantoic fluid. The absorption by the yolk and albumen was only passive and was low enough to be unimportant. At this time there was comparatively little difference in P^{32} between the tumor and embryo skeleton. The skeleton continued to accumulate P^{32} more rapidly than the tumor and at 96 hours contained about 5 times as much. The amount in the blood dropped rapidly, due to its absorption by the tissues. There was a slight drop in the P^{32} found in the allantoic fluid, since the reabsorption of this liquid into the system had begun by this time.

Table 2 illustrates the results of an experiment

TABLE 2

COMPARISON OF P^{32} DISTRIBUTION IN TUMOR AND CHICK EMBRYO LIVER AND BRAIN

Material	Hours after P^{32} injection	Counts/minute/ 100 mg dry wt.	Ratio
Chick embryo liver	48	398	1.0
Chick embryo brain	48	928	2.3
C3H tumor	48	1,300	3.2

comparing the uptake of P^{32} by the C3H mouse tumor, chick embryo brain, and chick embryo liver; 48 hours after injection of P^{32} the tumor has taken up more than either the brain or the liver.

CONCLUSIONS

In the use of egg-grown tumors for chemotherapy it is important to know the manner in which the chemotherapeutic substances are taken up and distributed. While this process will not be identical for all such substances, a general idea may be obtained from the results of these P^{32} experiments.

The P^{32} is taken up and distributed via the blood stream, as can be seen by the increase and then decrease of the amount present. While some of the P^{32} is absorbed by the various tissues, the greater part of it passes into the allantoic fluid. It is thus evident that before a substance is discarded as having no tumor-inhibiting effect, it is necessary to determine whether its impotency is due to rapid excretion or physiological inactivity. If it is the former, then another method of injection designed to prevent rapid excretion (e.g., yolk, oil solvents, etc.) is necessary.

The greater accumulation of P^{32} by the bones of the chick embryo than by the tumor is in agreement with results obtained in mice. Such a distribution of P^{32} would result in destruction of the bone marrow before enough could accumulate in the tumor to destroy it.

SUMMARY

1. A C3H mammary carcinoma was grown in fertile chicken eggs by the yolk sac injection technic.

2. Radioactive phosphorus in a 1 per cent Na_2HPO_4 solution was injected between the chorio-allantoic membrane and the egg shell 8 days after tumor inoculation.

3. The P^{32} was taken up by the blood stream and distributed throughout the egg. At 48 hours most of the P^{32} was found in the allantoic fluid. The tumor took up more P^{32} than either the chick embryo brain or liver. The chick embryo skeleton accumulated P^{32} more rapidly than the mouse tumor and at 96 hours after injection contained about 5 times as much.

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REFERENCES

1. TAYLOR, A., and CARMICHAEL, N. Yolk Sac Cultivated Tumor Tissue and Experiments in Tumor Chemotherapy. I. Riboflavin. Texas Rep. Biol. & Med., **6**:504–12, 1948.
2. TAYLOR, A.; CARMICHAEL, N.; and NORRIS, T. A Further Report on Yolk Sac Cultivation of Tumor Tissue. Cancer Research, **8**:264–69, 1948.

Studies on the Distribution of Radioactive 8-Azaguanine (Guanazolo) in Mice with Eo771 Tumors*†

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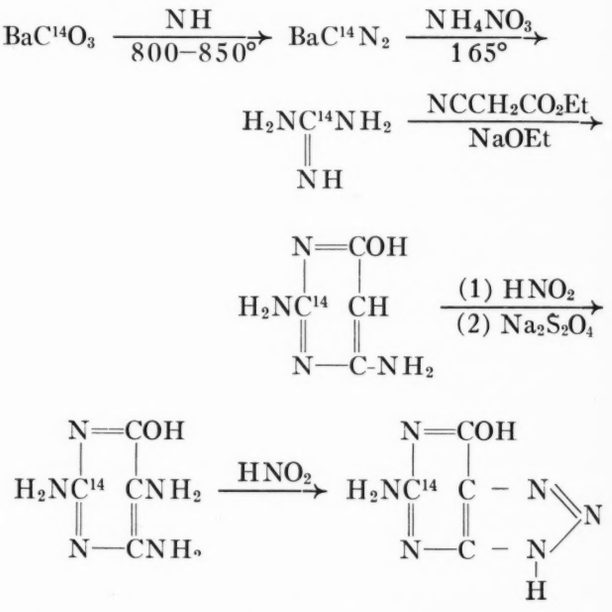
Kidder's observation (7) that 8-azaguanine (guanazolo) has a definite inhibitory effect on the growth of mammary adenocarcinoma Eo771 has now been extended and confirmed (5, 12). However, it has been found that Sarcoma 180, lymphosarcoma 6C3HED, sarcoma T241, Harding-Passey melanoma, Wagner osteogenic sarcoma or Patterson lymphosarcoma in the mouse, and certain carcinomas and sarcomas in the rat fail to respond to this compound (5, 12). 8-Azaguanine has also been shown to effect an inhibition of two transplantable leukemias in mice (8), while other strains failed to respond (1, 8). Kidder *et al.* (7) suggested that the tumor-inhibiting activity of 8-azaguanine might be explained on a basis of preferential incorporation of this compound into cancer cells because of differences in guanine metabolism. Roblin *et al.* (10) found 8-azaguanine to be a potent purine inhibitor in *Escherichia coli* and *Staphylococcus aureus*. Kidder (6) has reported a similar inhibition of growth in *Tetrahymena geleii*.

In view of the importance of such observations as have been mentioned above, it was considered worth-while to investigate the tumor-inhibiting mechanism of the action of 8-azaguanine, by use of the carbon 14-labeled compound. The synthesis of 8-azaguanine-2-C^{14} was accomplished by minor modifications of established synthetic methods, as follows. The final product was characterized by ultraviolet absorption spectrum, elemental analysis, and filter paper chromatography combined with autoradiography.



The present study concerns the over-all distribution of the 2-carbon atom of 8-azaguanine at various periods after injection into both normal mice and mice bearing a tumor known to respond —Eo771 adenocarcinoma. Further investigation of Kidder's hypothesis regarding preferential entrance of this purine antagonist into nucleic acids of tumor cells is being reported in the next article of this issue (9).

EXPERIMENTAL

SYNTHESIS OF RADIOACTIVE 8-AZAGUANINE

Guanidine hydrochloride.—Barium carbonate (3 mc., 168 mg.) was converted to guanidine hydrochloride essentially as described by Marsh, Lane, and Salley (3). The crude product (47 mg.) was diluted to 1 gm. with inactive guanidine hydrochloride and was used directly in the next step.

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2,4,5-Triamino-6-hydroxypyrimidine.—Guanidine hydrochloride (1 gm.) was converted to 2,4,5-triamino-6-hydroxypyrimidine by the procedure of Cain, Mallette, and Taylor (2), with slight modifications. The pyrimidine was isolated as the sulfate; the yield was 0.65 gm., 24 per cent from guanidine. In subsequent syntheses, this pyrimidine has been obtained in a 70–75 per cent yield from guanidine and in a 40–59 per cent over-all yield from barium carbonate.

8-Azaguanine.—The procedure of Roblin *et al.* (10) was used for conversion of 2,4,5-triamino-6-hydroxypyrimidine to 8-azaguanine. The crude product, after being twice dissolved in sodium hydroxide, treated with charcoal, and reprecipitated with acetic acid, weighed 257 mg. and had an activity of 0.97 μc/mg (67 per cent yield from 2,4,-5-triamino-6-hydroxypyrimidine; over-all yield from barium carbonate, 8.3 per cent).

Analysis: Calculated for $C_4H_4ON_6$: C, 31.58; H, 2.65
Found: C, 31.47; H, 2.82

The ultraviolet absorption spectrum, determined on a solution of 3.7 mg/l in phosphate buffer of pH 6.30, was identical with that reported by Cavalieri *et al.* (4).

As a further check on purity, the sodium salt was chromatographed (ascending) on filter paper with the following results:

Solvent System	R_F values Found	Reported*
n-C_4H_9OH (4 parts), diethylene glycol (1 part), H_2O (1 part) in an NH_3 atmosphere	0.11	0.13
n-C_4H_9OH (4 parts), diethylene glycol (1 part), 2 N HCl (1 part)	0.42	0.42–0.46

* A. Bendich, Sloan-Kettering Institute for Cancer Research. Private communication.

In an independent experiment the sodium salt was chromatographed, and an autoradiograph was made of the filter paper strip. The position of the radioactive spot coincided exactly with the position of the 8-azaguanine as determined in ultraviolet light; there was no detectable radioactivity elsewhere on the filter paper.

INJECTION OF RADIOACTIVE 8-AZAGUANINE

In a preliminary experiment, two normal CFW strain mice of about 6 weeks of age were each injected with 1.93 μc. of labeled 8-azaguanine (80 mg/kg), made up in 1 per cent sodium carbonate. These animals were placed immediately into a metabolism chamber (11) for collection of expired carbon dioxide and excreta. After 24 hours, the mice were killed, and selected pooled tissues and organs were assayed for radioactivity. The data obtained in this experiment with regard to excretion and distribution of radioactive carbon are summarized in Tables 1 and 2.

TABLE 1

RATE OF EXPIRATION AND URINARY EXCRETION OF C^{14} FOLLOWING INJECTION OF LABELED 8-AZAGUANINE (1.93 μc.)

Period	Specific activity of samples (μc/mole carbon)*	Per cent of total injected radioactivity	Cumulative per cent of total injected radioactivity
0–30 minutes	0.644	0.06	0.06
30–60 minutes	0.663	0.08	0.14
1–2 hours	0.096	0.02	0.16
2–6 hours	0.015	0.01	0.17
6–12 hours	0.142	0.03	0.20
12–24 hours	0.107	0.15	0.35
0–24-hour urine sample	321.0	91.4	

* Microcuries per mole of carbon.

NOTE: These are the average data obtained on pooled respiratory samples from two normal CFW strain mice.

TABLE 2

GENERAL DISTRIBUTION OF RADIOACTIVE CARBON AT VARIOUS PERIODS AFTER INJECTION OF 2-LABELED 8-AZAGUANINE

SPECIFIC ACTIVITY (μc/MOLE OF CARBON)*

Tissue	Normal CFW strain mice 24 hrs. after injection	C57 black mice with Eo771 Tumors Period after injection 1 hr.	6 hrs.	24 hrs
Whole blood	0.012	0.392	0.183	0.016
Blood serum	0.099			
Blood cells	0.016			
Spleen	0.079	0.776	0.467	0.131
Adrenals		0.389	0.125	0.012
Kidneys	0.086			
Liver	0.041	0.503	0.350	0.078
Testes	0.036			
Thymus	0.054			
Lungs	0.025			
Heart	0.056			
Lymph nodes	0.037			
Muscle	0.008	0.828	0.030	0.019
Jejunum	0.157	1.170	0.344	0.306
Skin and hair	0.018			
Whole bone	0.036			
Bone marrow	0.089	0.594	0.129	0.028
Eo771 tumor (animal 1)		0.861	0.130	0.078
Eo771 tumor (animal 2)		0.432	0.167	0.074
Urine	321.0			
Feces	8.15			

* Microcuries per mole of carbon.

A second series of investigations was then undertaken to determine the distribution of carbon 14 from labeled 8-azaguanine in C57 black mice with the Eo771 adenocarcinoma. Subcutaneous implantations of small pieces of tumor (measuring about 1.5 mm. cubed, and weighing approximately 6 mg.) were made in the axillary region by the usual trocar method. After 8 days,

two of these tumor-bearing mice were each injected with 1.93 μc. of radioactive 8-azaguanine. One hour after the injection of the radioactive compound, these mice were sacrificed for tissue and organ activity assays. The tissues of interest from the two mice used in each of these experiments were pooled, except in the case of the tumors which were run separately. Two additional mice (9 days after tumor implantation) were injected at the same level of labeled 8-azaguanine and were sacrificed for distribution studies at 6 hours. A similar study was carried out on two Eo771 tumor-bearing mice (9 days after implantation), in which mice were sacrificed for activity studies 24 hours after injection of the labeled compound. The results obtained in these experiments are presented in Table 2.

DISCUSSION

The results presented in Table 1 indicate the route of excretion of the 2-carbon atom of 8-azaguanine. Since only about 0.35 per cent of the total injected radioactive carbon was found in the exhaled carbon dioxide, it appears that there was no extensive oxidation of the molecule. Most of the activity injected (91.4 per cent) can be accounted for in the urine within 24 hours.

The distribution of the active carbon atom in CFW mice at 24 hours shows more activity in the blood serum than in packed cells and the highest specific activity in the jejunum. Otherwise, the active carbon is generally found in all tissue fractions.

In the Eo771 tumor-bearing C57 black mice the specific activities of the tumors fall within the range covered by the other tissues. The jejunum is again the highest in radioactivity, in all cases being higher than the tumor tissue from the same animals. Although the present data do not indicate preferential accumulation of 8-azaguanine in neoplastic tissue, the possibility obviously exists that 8-azaguanine might be preferentially fixed to or incorporated into important fractions of cancer cells, i.e., nucleic acids, which might be masked in these gross observations. This point has received special attention in experiments reported in the following article (9).

SUMMARY

Studies with 2-labeled 8-azaguanine (guanazolo) in normal and mammary adenocarcinoma-bearing mice have shown that very little of the 2-carbon atom is oxidized to carbon dioxide and exhaled, but that most of this atom is excreted in the urine. No evidence was obtained that the compound in question (or the 2-carbon therefrom) was preferentially accumulated, fixed, or incorporated in Eo771 tumors, which are known to be inhibited by 8-azaguanine.

REFERENCES

1. Burchenal, J. H.; Johnston, S. F.; Burchenal, J. R.; Kushida, M. N.; Robinson, E.; and Stock, C. C. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **71**:381–87, 1949.
2. Cain, C. K.; Mallette, M. F.; and Taylor, E. C. Pyrimido (4, 5-b) Pyrazines. I. Synthesis of 6,7-Symmetrically Substituted Derivatives. J. Am. Chem. Soc., **68**:1996–99, 1946.
3. Calvin, M.; Heidelberger, C.; Reid, J. C.; Tolbert, B. M.; and Yankwich, P. F. Isotopic Carbon, pp. 158–59. New York: John Wiley & Sons, Inc., 1949.
4. Cavalieri, L. F.; Bendich, A.; Tinker, J. F.; and Brown, G. B. Ultraviolet Absorption Spectra of Purines, Pyrimidines and Triazolopyrimidines. J. Am. Chem. Soc., **70**:3875–80, 1948.
5. Gellhorn, A.; Engelman, N.; Shapiro, D.; Graff, S.; and Gillespie, H. The Effect of 5-Amino-7-Hydroxy-1*H*-*v*-triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals. Cancer Research, **10**: 170–77, 1950.
6. Kidder, G. W., and Dewey, V. C. The Biological Activity of Substituted Pyrimidines. J. Biol. Chem., **178**:383–87, 1949.
7. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Woodside, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
8. Law, L. W. Studies on the Effects of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
9. Mitchell, J. H., Jr.; Skipper, H. E.; and Bennett, L. L., Jr. Investigation of the Nucleic Acids of Viscera and Tumor Tissue from Animals Injected with Radioactive 8-Azaguanine. Cancer Research, **10**:647–49, 1950.
10. Roblin, R. O., Jr.; Lampen, J. O.; English, J. P.; Cole, Q. P.; and Vaughan, J. R., Jr. Studies in Chemotherapy. VIII. Methionine and Purine Antagonists and Their Relation to the Sulfonamides. J. Am. Chem. Soc., **67**:290–94, 1945.
11. Skipper, H. E.; Bryan, C. E.; White, L., Jr.; and Hutchison, O. S. Techniques for *in Vivo* Tracer Studies with Radioactive Carbon. J. Biol. Chem., **173**:371–81, 1948.
12. Sugiura, K.; Hitchings, G. H.; Cavalieri, L. F.; and Stock, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**: 178–85, 1950.

Investigation of the Nucleic Acids of Viscera and Tumor Tissue from Animals Injected with Radioactive 8-Azaguanine*

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In view of the interest in the tumor growth-inhibiting property of 8-azaguanine (5-amino-7-hydroxy-1*H*-*v*-triazolo [*d*] pyrimidine [4, 5, 10]), it became of interest to learn more about the mechanism of its action. Kidder and Dewey (6) reported that *Tetrahymena geleii* requires guanine in its diet and that 8-azaguanine is a powerful inhibitor (7), which was thought to produce its effect by being incorporated in the nucleoprotein. It has been shown (2) that rats can synthesize guanine from adenine but that they do not extensively make use of dietary guanine. Kidder *et al.* (5) theorized that, since certain tumors (Eo771) were inhibited in growth by 8-azaguanine, perhaps cancer cells had lost the ability to synthesize guanine and had gained the ability to metabolize it.

We have synthesized 8-azaguanine (1) labeled with carbon 14 in the 2-position and have followed its incorporation (1) into the principal tissues of the mouse (CFW and C57 black with Eo771 tumors). The jejunum was shown to be highest in radioactivity of all tissues studied, including the tumors.

In this paper we present evidence to show that the nucleic acid fractions isolated from normal viscera and from Eo771 tissue of mice injected with carbon 14-labeled 8-azaguanine contain small amounts of 8-azaguanine.

EXPERIMENTAL

Injection of radioactive 8-azaguanine.—CFW mice were injected with 2 mg. each of 8-azaguanine labeled with carbon 14 in the 2-position and with an activity of 0.97 μc. per milligram. C57 black mice were injected with the same amount of carbon 14-labeled 8-azaguanine 8 days after subcutaneous implantations of small pieces of Eo771 tumor tissue.[1] Groups of four mice each were utilized in each experiment.

Isolation of nucleic acids and purines.—Twenty-four hours after injection of the radioactive compound, the animals were killed, and liver, spleen, thymus, gonads, kidneys, and washed intestine were combined for isolation of the nucleic acids of the viscera.

The tissue was disintegrated in a Waring Blendor and dehydrated with several portions of absolute ethanol and ethyl ether. The dehydrated tissue was extracted with 10 per cent NaCl, and the sodium salts of the nucleic acids were precipitated with alcohol, dissolved in water, and the nucleic acids precipitated with HCl, washed with water, and dried with alcohol and ether (8). To obtain DNA, the nucleic acids were twice hydrolyzed for about 8 hours at room temperature in a solution of approximately 2 N NaOH and reprecipitated in acid alcohol (3).

Analyses of the nucleic acid fractions by the orcinol and diphenylamine color reactions showed that the combined nucleic acids and DNA were more than 90 per cent pure.

The nucleic acids were hydrolyzed in 0 5 N HCl for 1 hour, and the purines were precipitated in acid solution with $AgNO_3$. The purines were redissolved in 0.5 N HCl and reprecipitated with $AgNO_3$ in acid solution.

Chromatographic separation of purines.—The purines were extracted from their silver compounds with 6 cc. of 0.5 N HCl, and 10 mg. of

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[1] These tumor-bearing mice were generously supplied by Dr. K. Sugiura of the Sloan-Kettering Institute for Cancer Research.

inactive 8-azaguanine was added to the solution as a carrier for radioactive 8-azaguanine which might be present. This solution was applied as a series of drops from a 1-ml. hypodermic syringe to the bottom of a sheet of 18×18-inch Schleicher and Schüll No. 597 filter paper. The drops were so spaced as to form a continuous band across the paper.

The solvent system used for separation of the purines (11) consisted of 4 parts butanol, 1 part diethylene glycol, and 1 part aqueous 0.1 N HCl, and the separation was achieved in 18 or 20 hours by the ascending method. After drying in the air for several hours and in an oven at 90° C. for 30 minutes, the adenine, guanine, and 8-azaguanine strips were cut from the paper in the light of a Mineralight ultraviolet lamp. This light is strongly absorbed by the above compounds, making the location of each easily discernible. The R_F values for guanine, adenine, and 8-azaguanine in this solvent system were 0.12, 0.21, and 0.43, respectively. These values agree well with those obtained by Bendich.[2]

The 8-azaguanine strip and adenine strip were eluted separately with 0.1 N NH_4OH, while the guanine strip was eluted with 0.1 N KOH. Adenine was precipitated from solution as the picrate by adding an equal volume of saturated picric acid. To the 8-azaguanine and guanine solutions were added 10 mg. of nonradioactive 8-azaguanine and guanine, respectively, and these purines were then precipitated as their silver salts.

Determination of radioactivity.—Measurements for radioactivity were made as previously described (9).

RESULTS AND DISCUSSION

Table 1 shows the radioactivity of the original tissue (initial homogenate), the combined nucleic acid fraction, DNA, and silver purines from mice 24 hours after each animal was injected with 2 mg. of 8-azaguanine labeled at the 2-position with carbon 14 and with an activity of 0.97μc. per milligram.

The combined nucleic acid fractions have several times more activity than the viscera from which they were isolated, while the DNA is low in activity. The silver purines have an activity about 4 times higher than the combined nucleic acid fraction from which they were isolated.

The nucleic acid fractions isolated from Eo771 tumor tissue were somewhat lower in radioactive carbon than the visceral nucleic acid fractions from the same animals.

2. A. Bendich, Sloan-Kettering Institute for Cancer Research. Private communication.

Inasmuch as it was desirable to determine whether or not the method for nucleic acid isolation also resulted in concentrating any "free" 8-azaguanine which might be present, an amount of this compound was added to viscera from injected animals. The nucleic acids isolated from this preparation were at least 20 times lower in radioactivity than nucleic acids from injected animals (Table 1), thus showing that the method used for isolating the nucleic acid fractions does not concentrate "free" 8-azaguanine.

TABLE 1

RADIOACTIVITY OF FRACTIONS FROM MICE INJECTED WITH RADIOACTIVE 8-AZAGUANINE*

EXPER. NO.	SAMPLE DESIGNATION	SPECIFIC ACTIVITY, μc. PER MOLE OF CARBON: Initial homogenate	Combined nucleic acids	DNA	Silver purines
1	Black mice with Eo771 Tumor:				
	Viscera	0.12	1.06	0.02	
	Tumor	0.10	0.37		
2	Black mice with Eo771 Tumor:				
	Viscera		1.10		3.93
	Tumor		0.70		
3	CFW mice	0.14		0.12	
5	CFW mice	0.18	0.83	0.19	
6	CFW mice	0.29	0.68	0.13	
7	Radioactive 8-azaguanine added to inactive tissue	0.12	0.03		

* All animals were sacrificed 24 hours following injection. Each experiment represents values obtained on pooled organs or tissues from four mice.

TABLE 2

RADIOACTIVITY OF PURINES FROM MICE INJECTED WITH CARBON-LABELED 8-AZAGUANINE

EXPER. NO.	SAMPLE DESIGNATION	SPECIFIC ACTIVITY, μc. PER MOLE OF CARBON: Initial homogenate	Combined nucleic acids	Adenine	Guanine	8-Azaguanine
8*	CFW mice	0.29	0.96	0.12	0.09	1.34 (x)
9†	CFW mice	0.08	0.72	0.26	0.59	2.16 (x)

* Animals were sacrificed 6 hours following injection.

† Animals were sacrificed 24 hours following injection.

NOTE: The adenine and guanine specific activities have been corrected for dilution with inactive carrier; the 8-azaguanine values have not been corrected. A correction factor (x) in excess of 15,000 is estimated.

Table 2 shows the radioactivity of the purines obtained from combined nucleic acid fractions. The values for adenine and guanine have been corrected for dilution, while the values for 8-azaguanine must be multiplied by a large factor (x = at least 15,000), since the dilution with inactive 8-azaguanine is great. These results indicate that a small amount of the injected 8-azaguanine is combined in the nucleic acid structure as suggested by Kidder *et al.* (5), or that it is isolated along with the nucleic acid fraction in some other

fashion, possibly as an enzyme-8-azaguanine complex.

Assuming for the purpose of calculation that all the radioactivity of the combined nucleic acids is due to 8-azaguanine, it is possible to estimate that the maximum quantity of 8-azaguanine contained in the visceral nucleic acid fraction isolated from one mouse is about 0.5 μg.

It would appear that the presence of radioactive carbon (from 2-labeled 8-azaguanine) in the adenine and guanine of visceral nucleic acids (Table 2) could only be accounted for by utilization of degradation products containing the active atom in synthesis of the naturally occurring purines.

SUMMARY

1. Radioactive carbon from carbon-labeled 8-azaguanine, at 6 and 24 hours after injection, is found in higher concentrations in the nucleic acid fractions of normal viscera and of Eo771 tumor tissue than in the tissues from which the nucleic acids were isolated.

2. The nucleic acid fraction from Eo771 tumor tissue is no higher in radioactivity than the nucleic acid fraction from normal visceral tissue.

3. 8-Azaguanine has been shown to be present in the purines isolated from the ribosenucleic acid fraction of visceral tissue of animals injected with radioactive 8-azaguanine.

REFERENCES

1. BENNETT, L. L., JR.; SKIPPER, H. E.; MITCHELL, J. H., JR.; and SUGIURA, K. Studies on the Distribution of Radioactive 8-Azaguanine (Guanazolo) in Mice with Eo771 Tumors. Cancer Research, **10**:644–46, 1950.
2. BROWN, G. B.; ROLL, P. M.; PLENTL, A. A.; and CAVALIERI, L. F. The Utilization of Adenine for Nucleic Acid Synthesis and as a Precursor of Guanine. J. Biol. Chem., **172**:469–84, 1948.
3. BRUES, A. M.; TRACY, M. M.; and COHN, W. E. Nucleic Acids of Rat Liver and Hepatoma: Their Metabolic Turnover in Relation to Growth. J. Biol. Chem., **155**:619–33, 1944.
4. GELLHORN, A.; ENGELMAN, M.; SHAPIRO, D.; GRAFF, S.; and GILLESPIE, H. The Effect of 5-Amino-7-Hydroxy-1*H*-*s*-Triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals. Cancer Research, **10**:170–77, 1950.
5. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
6. KIDDER, G. W., and DEWEY, V. C. Biochemistry of Tetrahymena. XIV. Activity of Natural Purines and Pyrimidines. Proc. Nat. Acad. Sc., **34**:566–74, 1948.
7. ———. The Biological Activity of Substituted Purines. J. Biol. Chem., **179**:181–87, 1949.
8. PLENTL, A. A., and SCHOENHEIMER, R. Studies in the Metabolism of Purines and Pyrimidines by Means of Isotopic Nitrogen. J. Biol. Chem., **153**:203–17, 1944.
9. SKIPPER, H. E.; BRYAN, C. E.; WHITE, L., JR.; and HUTCHISON, O. S. Techniques for *in vivo* Tracer Studies with Radioactive Carbon. J. Biol. Chem., **173**:371–81, 1948.
10. SUGIURA, K.; HITCHINGS, G. H.; CAVALIERI, L. F.; and STOCK, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**:178–85, 1950.
11. VISCHER, E., and CHARGAFF, E. The Separation and Quantitative Estimation of Purines and Pyrimidines in Minute Amounts. J. Biol. Chem., **176**:703–14, 1948.

A Comparison of the Intracellular Composition of Regenerating Liver and Induced Liver Tumors*†

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Rat liver tumors induced by 4-dimethylaminoazobenzene differ considerably from normal rat liver with respect to the distribution of protein, nucleic acids, and other constituents among the morphological fractions of their cells (21, 24, 27). One of the gross differences between these tissues is the rapid growth of the tumor tissue, and it may be that the differences in intracellular composition are related to the more rapid rate of growth of the tumors. Since regenerating liver cells grow at a rate which probably even exceeds that of liver tumors, it seemed of interest to investigate the intracellular distribution of nucleic acids and protein in regenerating liver, and to compare the composition of these cells with that of normal and neoplastic liver cells.

Novikoff and Potter (19) found a considerable increase in the pentosenucleic acid content of unfractionated regenerating liver during the period of rapid gain in weight. The maximum levels were found at 2–3 days after partial hepatectomy. They did not find any consistent change, with time, in the desoxypentosenucleic acid content of the liver after partial hepatectomy. Using an ultraviolet cytochemical technic, Stowell (29) found little change in the nucleic acid concentration in the first day of regeneration, but during the second day, when there was rapid cell division, there was an increase in the nucleic acid concentration in the nucleolus and in the cytoplasm adjacent to the nucleus. The experiments reported here were designed to determine in which fractions of the cytoplasm the changes observed by the previous authors occurred.

For several reasons, the data have been expressed in terms of units of constituent per cell, the number of cells having been determined by counting nuclei. Brues, Drury, and Brues (3) demonstrated that there was no significant increase in the number of hepatic cells during the first day of liver regeneration, although the liver increased in weight by 50–60 per cent. It has also been shown that the nucleocytoplasmic ratio is different from normal in both regenerating (29) and hepatoma cells (26, 30). These types of cells also differ from normal liver cells with respect to size (30). Stowell (29) found that the increase in cell size during the early phases of regeneration is accompanied by an increase in the stainable fat in the cytoplasm, as well as a decrease in the vascular spaces in the tissue. It has also been shown that there is a considerable amount of blood in the liver of a rat after death, and that the amount varies with the method of killing the animal (27). Factors such as these indicate that there may be considerable differences in the numbers of cells per unit weight of fresh liver. Hence, since we were interested in the changes that occurred in the average liver cell, it was felt that it was better to express the results in terms of units per nucleus than in terms of fresh or dry weight of tissue.

Studies by Boivin, Vendrely, and Vendrely (2), Mirsky and Ris (18), Price, Miller, Miller, and Weber (23), and Dounce, Tishkoff, Barnett, and Freer (9) have shown the advantages of considering the nucleus as a unit in studying the desoxypentosenucleic acid content of various tissues. Davidson and Leslie (8) have recently applied this concept to biochemical studies of tissue culture growth and have used desoxypentosenucleic acid as a standard of reference to reveal changes in the amount of other cell constituents.

METHODS

Male albino rats[1] weighing 195–215 gm. were fed a stock grain diet (16) *ad libitum* throughout the experiments. The partial hepatectomies were performed by the method of Higgins and Anderson

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† We are indebted to Drs. E. C. and J. A. Miller for their advice and continued interest in this project. We also wish to thank Dr. Hans Ris for his assistance in the Feulgen staining and his advice during the course of this work.

‡ Postdoctoral Research Fellow, Public Health Service, 1949–50.

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[1] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

(12). The animals were killed with ether, and the livers were removed, weighed, and perfused with 0.14 M sodium chloride as previously described (27). Livers from four animals were used for each fractionation. The pooled livers were forced through a plastic tissue mincer, homogenized in 0.88 M sucrose, and fractionated by differential centrifugation into nuclear, large granule, small granule and supernatant fluid fractions, as previously described (20, 22, 24). Each fraction and the unfractionated homogenates were analyzed for nucleic acids (25) and protein nitrogen (32). The protein was prepared for digestion by precipitation with 10 per cent trichloroacetic acid, followed by washing the precipitate twice with 5 per cent trichloroacetic acid, twice with cold 95 per cent ethanol, 3 times with hot 3–1 ethanol-ether, once with cold ethyl ether, and then by drying at room temperature. Nuclei in the unfractionated homogenate were enumerated with a hemocytometer by the method previously described (23). The counts were made independently by two persons, and, if necessary, they were repeated until a difference of less than 5 per cent was obtained. Blocks of all livers were fixed in Zenker's and Mossman's fixatives (11). The fixed tissues were imbedded in paraffin, sectioned, and stained with hematoxylin and eosin (14). Homogenates were fixed in Helly's fixative (14), as previously described (23), and stained by the Feulgen technic (14). Three separate groups of animals were used. The livers of the rats of the first group were fractionated 1, 2, 3, 4, 7, and 23 days after partial hepatectomy. The livers of the second group were fractionated after 1, 2, 3, 4, 6, and 8 days of regeneration. The livers of the third group were not fractionated, but the whole homogenates were analyzed for nucleic acids. Two homogenates of the livers of the third group of animals were prepared, one in the sucrose medium, and the other in 1 per cent citric acid. The latter homogenate was used only for the enumeration of nuclei, and the values were compared with those obtained in the sucrose homogenates, since it has been shown that nuclei treated with citric acid are very resistant to fragmentation (17, 28), and it is possible that the nuclei of regenerating liver cells might be more fragile than those of normal liver. These animals were killed after $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, 1, 2, 3, 4, 6, and 8 days of regeneration. The results obtained in the two series of fractionations were in good agreement, and those of the second series will be presented in detail.

The data for tumor tissue were taken from a previous publication (24) and recalculated on the assumption that the tumors contained 10.0×10^{-12} gm. of desoxypentosenucleic acid in the average nucleus; this figure was found in unpublished experiments.[2] In converting tumor protein to protein nitrogen, the tumor proteins were assumed to contain 16 per cent of nitrogen. The errors introduced by these assumptions are probably insignificant in comparison with the great differences between the tumor cells and the normal liver cells.

RESULTS

Nuclei per gram of liver.—The control livers contained 131,000,000 nuclei per gram of fresh tissue (Fig. 1). This is in good agreement with the data of Price, Miller, Miller, and Weber (23), and

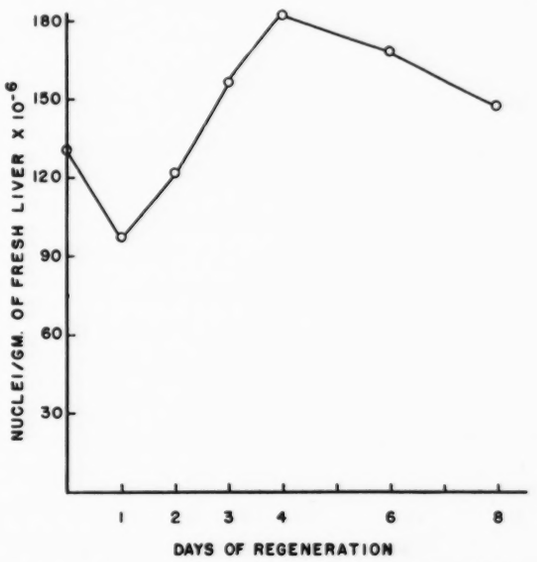

FIG. 1.—Changes in the numbers of nuclei found per gram of fresh liver during regeneration. In this and all subsequent figures each point represents a pool of 4 rat livers.

with those of Brues, Drury, and Brues (3) for normal rat liver. The number of nuclei was reduced to 98 million per gram after 1 day of regeneration, increased rapidly during the next 3 days to a maximum of 183 million, and then fell to 153 million by the eighth day. In the first experiment it was found that by the 23d day the number of nuclei per gram of fresh tissue equaled that of the controls. These changes in the numbers of nuclei are in agreement with data recalculated from Landing, Seed, and Banfield (13) for corresponding periods of time after partial hepatectomy. The changes in the weight of the average cell are probably due in large part to the variations in fat content and in the size of cells which have been shown to occur during regeneration (29). Such alterations in fat were readily apparent during the differential centrifugations, which brought a layer of fat to the top of the tubes. The thickness of this

[2] A. K. Laird and J. M. Price. Unpublished data.

layer decreased after the first day, until by the fourth day essentially no fat was visible.

The numbers of nuclei found in the sucrose and citric acid homogenates were in good agreement in all cases. In addition, it was not possible to

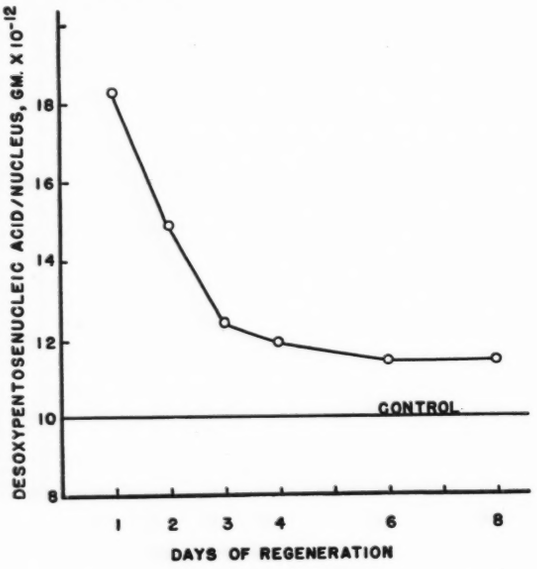

FIG. 2.—Desoxypentosenucleic acid content of the average nucleus at various times after partial hepatectomy. It should be noted that in this curve the ordinate starts at 8 units.

demonstrate Feulgen-positive particles other than nuclei in the fixed and stained homogenates of either normal or regenerating livers. In every case the desoxypentosenucleic acid of normal and of regenerating liver was found exclusively in the nuclear fraction. For these reasons it was felt that few, if any, nuclei were broken during homogenization.

Desoxypentosenucleic acid per nucleus.—At 1 day after partial hepatectomy, the desoxypentosenucleic acid content of the average nucleus had increased from a level of 10.0 to 18.3 $\times$ 10^{-12} gm. per nucleus (Fig. 2). In the third experiment, when the desoxypentosenucleic content of the average nucleus was determined at 6, 12, 18, and 24 hours after partial hepatectomy, it was found that the earliest significant increase was at 12 hours, and the points for 12, 18, and 24 hours fell on a straight line. During the next 2 days there was a remarkable decrease in the amount of desoxypentosenucleic acid per nucleus, and by the 23d day the normal level was reached. Essentially the same results were obtained in the other two experiments, except that the values for 1 day were not quite as high, falling between 15 and 16 $\times$ 10^{-12} gm. per nucleus.

It is of interest that in normal rat liver and in rat liver tumors induced by 4-dimethylaminoazobenzene Mark and Ris (15) found that nuclei of similar size contained the same amounts of desoxypentosenucleic acid. Laird and Price[2] and Cunningham *et al.* (7) have likewise found that various liver tumors and liver nuclei contain the same amount of desoxypentosenucleic acid.

It must be emphasized that the values obtained in this study represent the desoxypentosenucleic acid content of an average nucleus, and that because of polysomaty this is an intermediate value depending on the number of nuclei in each size class.

Protein nitrogen.—After 1 day of regeneration, the protein nitrogen of the average cell increased by about 17 per cent, largely because of increases in the nuclear and small granule fractions (Fig. 3). During the next 3 days the protein nitrogen fell to about 68 per cent of that of the normal livers. The largest decreases occurred in the large and small granules, where the levels at 4 days were about one-half of those of the normal livers. The nitrogen content of the supernatant fluid fraction also decreased, but not until after the third day. Only the nuclear fraction contained more protein nitrogen at all times during regeneration than was found in the corresponding fraction of the normal

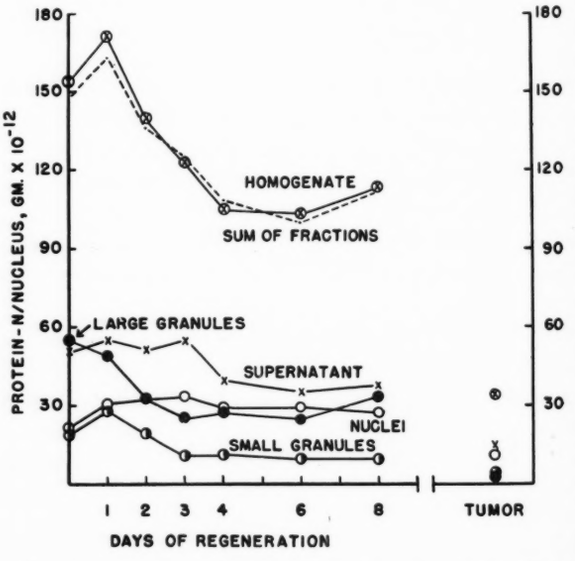

FIG. 3.—The intracellular distribution of protein nitrogen in normal and regenerating liver compared with that of tumors induced by 4-dimethylaminoazobenzene. The dashed line designated "Sum of Fractions" represents the sum of the amounts of protein nitrogen found in the four fractions, and approximates the curve for the unfractionated tissue or "Homogenate" according to the completeness of the recoveries.

liver. After 8 days of regeneration the other fractions all contained subnormal levels of protein nitrogen. By the 23d day all of the fractions were normal with respect to nitrogen content, except for the nuclear fraction, which was about 16 per cent above normal. The dashed line in Figure 3

represents the sum of the amounts of protein in the fractions.

The tumor cells, which are much smaller than the normal and regenerating liver cells (30), contained only about 23 per cent as much protein nitrogen as the normal liver cells, and only 34 per cent as much as regenerating liver cells at the point of minimum nitrogen content. The nuclei and large granules, however, contain about 55 and 6 per cent, respectively, as much nitrogen per cell as normal liver. The nuclei and large granules contained 40 and 12 per cent as much nitrogen, respectively, as the same fractions of regenerating liver cells 4 days after hepatectomy. Thus, the average tumor cell contained much less protein nitrogen than the average liver cell, either normal or during regeneration, and the relative amounts in the various fractions were quite different. This was particularly true of the large granule fraction, which was very low in protein nitrogen in the tumor tissue.

Pentosenucleic acid distribution.—The pentosenucleic acid content of the various fractions is shown in Figure 4. The pentosenucleic acid in the average liver cell increased by about 62 per cent in the first day of regeneration. In another experiment, it was found that this increase started 12 hours after partial hepatectomy, at the same time that the desoxypentosenucleic acid started to increase. This increase in pentosenucleic acid was the result of increases in the pentosenucleic acid of each fraction, but the increase in the small granules and supernatant fluid was much greater than in the other two fractions (93 and 125 per cent, respectively). The total pentosenucleic acid remained high on the second day, and then fell rapidly to almost normal by the sixth day. There was a slight rise in the pentosenucleic acid in the three particulate fractions between the sixth and eighth days. This increase was not observed in the first experiment in which analyses were made at 4 and 7 days, and after 23 days the levels were just slightly above the control values. It is of interest that the large granules changed very little with respect to pentosenucleic acid content during regeneration. The pentosenucleic acid content of the nuclei reached a peak after 3 days, and did not return to normal by the eighth day. The small granules and supernatant fluid curves were very similar, and accounted for most of the alterations in pentosenucleic acid content. The dashed line in Figure 4 represents the sum of the amounts of pentosenucleic acid in the four fractions.

Although the average tumor cell contained only about 36 per cent as much pentosenucleic acid as the average liver cell, the supernatant fluid fraction of the tumor cells contained slightly more of this component than normal liver cells. It was previously observed that this fraction of tumor tissue was particularly rich with respect to pentosenucleic acid (24). The nuclear fraction of tumors was likewise high in pentosenucleic acid content. When the data were calculated in terms of the amount per nucleus, however, it was apparent that the average tumor nucleus contained less of this nucleic acid than the average normal liver nucleus (Fig. 4). The pentosenucleic acid contents of the large and small granules of tumor tissue were very low (24), and the difference between normal liver and tumor tissue was much greater when the data were related to the nucleus as a unit (Fig. 4).

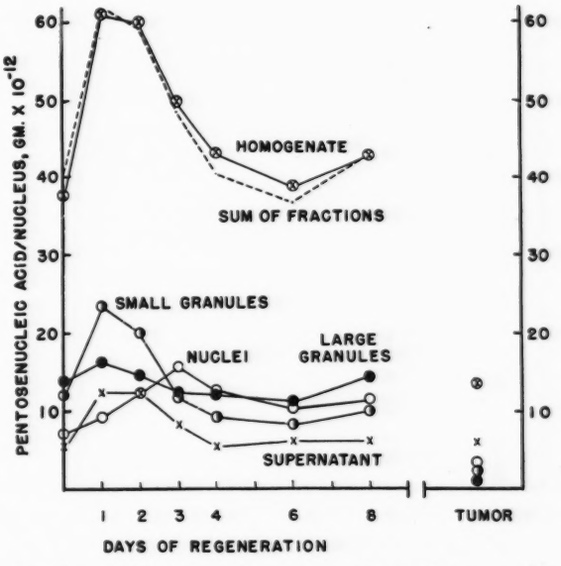

FIG. 4.—Distribution of pentosenucleic acid in normal and regenerating liver and in the liver tumors.

Ratio of pentosenucleic acid to protein nitrogen.—The ratio of pentosenucleic acid to protein nitrogen was greatly increased in all the fractions during regeneration (Fig. 5). This increase resulted from increases in the pentosenucleic acid and, after the first day, from decreases in the nitrogen content of the fractions. The ratios increased 109 per cent in the supernatant fluid by the first day and 69 per cent in the small granules by the second day. The ratios for the nuclei and large granules increased 42 and 93 per cent, respectively, on the third day of regeneration. Largely as a result of the incomplete return of protein nitrogen to normal by the eighth day, these ratios were elevated at this time. After 23 days, however, the ratio of pentosenucleic acid to protein nitrogen had returned to the control levels.

It was of interest that the ratio of pentosenucleic acid to protein nitrogen in the nuclear fraction and in the small granules was essentially the same in normal liver and tumor tissue. The same ratio in the large granules of tumor tissue

was between that of normal and the maximum attained in regenerating liver, while that of the supernatant fluid was 62 per cent higher than the maximum ratio reached in the same fraction of regenerating liver. This ratio in the supernatant fluid of the tumor is about 4 times that of the normal livers.

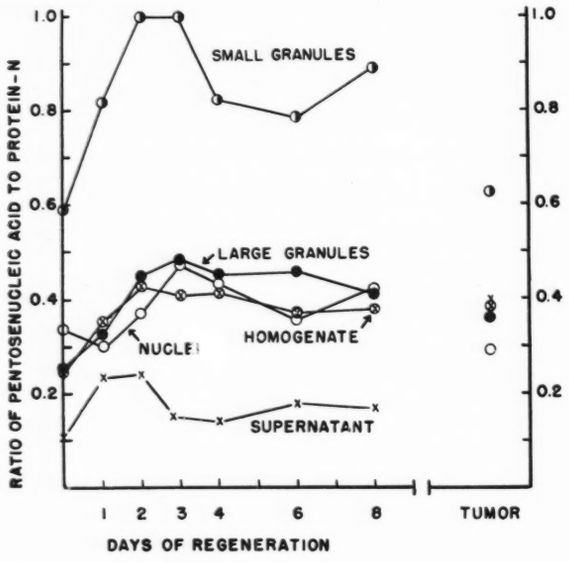

FIG. 5.—Ratio of pentosenucleic acid to protein nitrogen in normal and regenerating liver and in the liver tumors.

The increases in the ratios of pentosenucleic acid to protein nitrogen are in agreement with the results of Stowell (29), who used an ultraviolet absorption technic on individual cells. He noted an increase in the concentration of nucleic acid in the nucleolus shortly before there was a significant increase in the cytoplasm.

Comparison of the two methods of expressing the data.—Figure 6 shows the pentosenucleic acid in the homogenate and in the cytoplasmic granules expressed as units per nucleus and units per gram of fresh tissue. The scales are adjusted so that the comparable curves start at the same ordinate. The amount of pentosenucleic acid per average cell reached a maximum 1 day after partial hepatectomy, while the amount per gram of fresh tissue did not attain a maximum until after 3 days. The pentosenucleic acid per nucleus fell rapidly after 2 days, and by the sixth day had returned to essentially normal levels. At 6 days there was still a significant elevation of pentosenucleic acid on a fresh weight basis. There was likewise a great difference in the pentosenucleic acid contents of the cytoplasmic granules when the two methods of expressing the data were used. In the large granules the curves sloped in opposite directions during the first 4 days of regeneration. In the small granule fraction the biggest difference was in the time at which the maximum levels were reached, for the curves have roughly the same shape.

The most striking difference in the two methods of expression of the results was in the desoxypentosenucleic acid content of the nuclear fraction. During regeneration the desoxypentosenucleic acid content of a gram of fresh liver varied between 1.72 and 2.20 mg., with the maximum 4 days after the operation. In agreement with the observations of Novikoff and Potter (19), there was no evident relationship between the amount of desoxypentosenucleic acid in the fresh tissue and the period of regeneration. When the data were calculated with the nucleus as a unit, however, it was found that the average nucleus increased its desoxypentosenucleic acid content by 83 per cent before any evidence of increase in mitosis was found, and the subsequent period of rapid cell division coincided with a rapid fall in the desoxypentosenucleic acid per nucleus (Fig. 2).

Even more important in this regard was the comparison of the results of one experiment with another. Thus, when the results obtained with the second group of animals were compared on a fresh weight basis with those of the first group, the correspondence was not nearly as good as when compared on a cell basis. This is readily explained by the differences in the numbers of nuclei per gram of fresh liver in the two groups of rats at corresponding times after the hepatectomies, apparently from variations in the amount of fat, cell

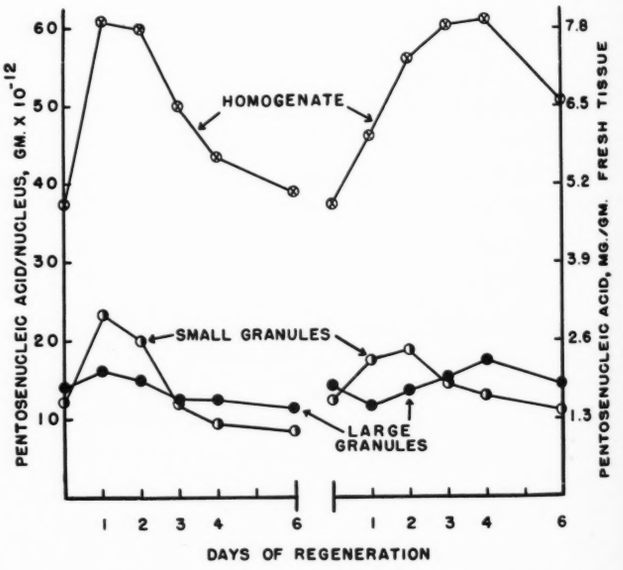

FIG. 6.—The pentosenucleic acid in the homogenate and cytoplasmic granules expressed as units per nucleus (left) and as units per gram of fresh liver (right). The scales were adjusted so that the curves started at the same ordinates. The other two fractions were omitted to preserve clarity and to facilitate comparison.

size, and similar factors. These variables are eliminated by using the nucleus as a unit of tissue.

Since rat liver contains at least 20 per cent binucleate cells (1, 4), one should not, strictly speaking, interchange the terms "cell" and "nucleus." However, Beams and King (1) were of the opinion that the binucleate cells of rat liver arise as the result of the failure of cytokinesis, and thus might be considered to be essentially the equivalent of two single liver cells in size and composition. Thus, it appeared that it was permissible to substitute "cell" for "nucleus" in certain instances for the sake of clarity, especially if the cell is thought of as a nucleus and its associated cytoplasm. However, due to the presence of polysomaty a significantly high percentage of rat liver cells (1, 31) will be larger than the diploid cells, and the average value of constituent per cell will be somewhat higher than the value for diploid cells.

Sham-operated controls.—One group of four animals was subjected to a laparotomy without the removal of any liver. Three days later the livers were fractionated and analyzed. The data were in good agreement with the data obtained from the control animals. The laparotomy apparently had a negligible effect on the results obtained.

Rate of regeneration.—Several rats were subjected to partial hepatectomy, immediately killed, and the remainder of the liver removed. The proportion of the liver removed by partial hepatectomy was found to be in good agreement with the results of other investigators (3, 13), which indicate that about two-thirds of the organ was removed. The rate of return of the liver to its preoperative weight was found to be in good agreement with the results of Novikoff and Potter (19), who used the same strain of rats and the same diet. Our results also confirmed the findings of Brues, Drury, and Brues (3) and Landing, Seed, and Banfield (13), who found that the liver regained its weight more rapidly than it regained its original number of cells, particularly during the first day after hepatectomy.

Histological and cytological observations.—A loose reticulation of the cytoplasm of many of the parenchymal cells was noted as early as 6 hours after hepatectomy. After 48 hours the reticulum was much denser, and minute basophilic particles were observed; concurrent with this change there was observed a localization of the cells so affected to the periphery of the lobule. In extreme cases the cells appeared quite empty, and only a rim of cytoplasm remained. This appearance of the cells and their localization within the lobule persisted in some livers as late as the eighth day after hepatectomy.

Mitoses were not observed up to and including 24 hours after hepatectomy; they reached a maximum frequency during the second and third days, and gradually became less numerous thereafter. During the first 24 hours the nuclei had a normal interphase appearance; later, all stages of mitosis could be seen.

Nuclei were classified according to size, in sections of control livers and of regenerating livers taken 24 hours and 48 hours after hepatectomy. It was found that about 33 per cent of the nuclei in control livers were of size classes larger than that corresponding to the diploid cells; of these only 0–4 per cent were of a size class corresponding to a level of polyploidy higher than tetraploid nuclei. In the 24-hour regenerating livers the percentage of large nuclei had risen to about 68 per cent, of which about 15 per cent were of a size class larger than the tetraploid. In the 48-hour livers the proportion of large nuclei had fallen almost to the control level. This increase in nuclear diameter agrees with the findings of Stowell (29). It is associated with the increase in nuclear size which occurs during prophase, rather than with polysomaty, since the nuclear diameters return toward the normal control value during the second 24 hours. It is of interest that the polyploid cells normally present in rat liver appear to share in the regenerative process. This is suggested by the finding that in the livers taken at 24 hours there was a sharp increase in the proportion of cells belonging to a size class larger than that corresponding to the tetraploid condition.

Cytoplasmic inclusion bodies were found in all livers during the first 48 hours after partial hepatectomy (Figs. 7 and 8). These bodies were round in section and variable in size, many reaching twice the diameter of the hepatic nuclei; in most cases they were sharply delimited from the cytoplasm by an optically empty space which surrounded them. Within many of the extremely large bodies found at 24 hours after hepatectomy, small, sharply defined, clear, yellowish vacuoles could be seen; these were not observed in sections taken before 24 hours.

To some extent the bodies took most of the stains tried, but in sections stained with hematoxylin and eosin they were clearly eosinophilic, and following the Gram stain they took the safranin counterstain very strongly. When stained with aniline acid fuchsin and methyl blue (6) following Regaud's fixative or picro-formol-acetic acid, they took the red stain in the center and the methyl blue in the periphery. They were present and comparable in appearance following fixation with Zenker-formol, picro-formol-acetic acid, Mossman's and Regaud's fixatives (11, 14), with

or without previous perfusion with physiological saline. They were Feulgen negative.

These structures were observed as early as 6 hours after hepatectomy; they reached maximum frequency at 24 hours, and had disappeared almost completely 48 hours after hepatectomy. In order

FIG. 7.—Liver removed 24 hours after partial hepatectomy. The numerous cytoplasmic inclusion bodies are distributed apparently at random throughout the lobules. (Picro-formol-acetic acid fixation, analine acid fuchsin-methyl blue stain.) Mag. ×150.

to rule out the possibility that the cytoplasmic inclusions were specific for the Holtzman strain of rats, livers of rats of the Long-Evans[3] and Sherman[3] strains were examined, and all were found to contain the bodies during the early regenerative period.

These intracytoplasmic structures are apparently similar to those described by Gurd and Vars (10), but these authors found them only in necrotic livers, whereas in the present series no gross or microscopic evidence of necrosis was found.

DISCUSSION

During the first 24 hours after partial hepatectomy the average liver cell showed an increase of 83 per cent in its desoxypentosenucleic acid content, while its pentosenucleic acid and protein nitrogen increased by 62 and 17 per cent, respectively. Since, by microscopic examination, dividing cells are first found soon after this time, it is probable that the cells doubled their desoxypentosenucleic acid and doubled their pentosenucleic acid in the small granule and supernatant fractions before dividing. As the cells divided, the nucleic acids were distributed among the daughter cells, and these new cells were never appreciably deficient in nucleic acids. The large granules increased relatively little in pentosenucleic acid before the division of cells took place but did not decrease appreciably during the rapid cell division. It would appear, therefore, that this fraction of the cell, unlike the other two cytoplasmic fractions, produced its pentosenucleic acid very near to or during the time of cell division, and then at a rate which was almost proportional to the rate of mitosis. This fraction of the cell appeared to lag behind the small granule and supernatant fluid fractions in pentosenucleic acid production. The average nucleus contained gradually increasing amounts of pentosenucleic acid until the third day, when the concentration of the latter started to return toward normal.

It was of interest that the increases in both nucleic acids started at about 12 hours after partial hepatectomy, and both reached maximum levels only 12 hours later. The numbers of cells in the livers did not double until between 24 and 48 hours after these maximum levels of nucleic acid were reached.

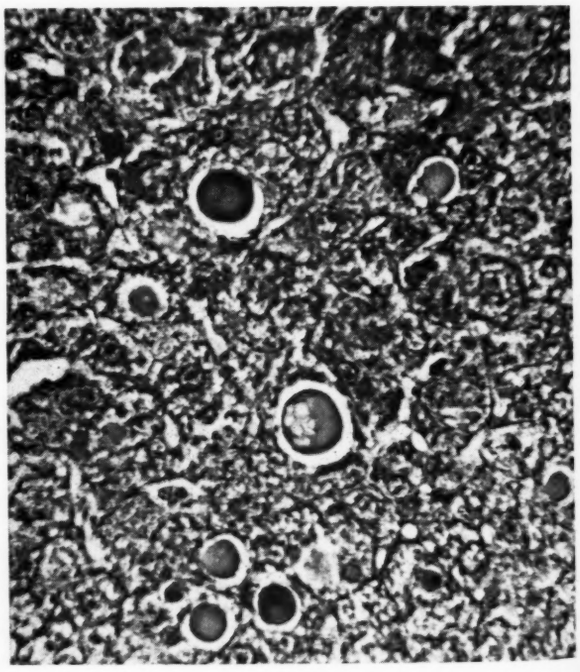

FIG. 8.—High power view of same liver shown in Figure 7, showing vacuoles within one of the bodies. Mag. ×360.

The fact that the nucleic acids increased to such high levels in the average cell before division started is an indication that most of the parenchymal cells begin the process of division at the same time. This would suggest the possibility of a humoral mechanism for the initiation of mitosis.

[3] Obtained from Rockland Farms, New City, New York.

Bucher, Scott, and Aub (5) obtained evidence for a blood-borne mechanism for control of liver cell mitoses when they found an increased rate of mitosis in the nonoperated parabiotic partner of a partially hepatectomized rat.

The rapid increase in pentosenucleic acid was not matched by the rate of protein nitrogen synthesis. There was an increase, in the first day, of 17 per cent, but thereafter the protein nitrogen content of the average cell fell rapidly to 68 per cent of normal by the fourth day. Thus, the average cell maintained its nucleic acid level at normal or above, while its protein nitrogen was not sustained. Between 8 and 23 days after partial hepatectomy the normal quantity of protein nitrogen in the average cell was restored.

The tumor cells are small compared to the normal or regenerating liver cells, so it was not surprising to observe the low levels of protein nitrogen and pentosenucleic acid in these cells. Calculation on a cellular basis, however, made possible some interesting comparisons. Thus, the average liver tumor cell had as much desoxypentosenucleic acid in its nucleus and as much pentosenucleic acid in its supernatant fluid as the normal liver cell. It was previously found that the pentosenucleic acid content of the supernatant fluid of liver tumors induced by 4-dimethylaminoazobenzene was relatively high (24). However, when the data are converted from units per gram of fresh tissue to units per cell it would appear that it is not elevated, but rather that all other fractions are exceedingly low, particularly the cytoplasmic granules.

The relative amounts of protein nitrogen and pentosenucleic acid in the various fractions of the neoplastic cells studied were quite different from either normal or regenerating liver. Therefore, it appears that we have three distinct tissues, and the neoplastic cells do not possess their peculiar composition merely because it is the optimum condition for rapid growth. The differences among the three types of tissue were also manifested in the ratio of pentosenucleic acid to protein nitrogen, where the nuclear and small granule fractions of tumor cells had ratios similar to normal liver. The large granule fraction had a ratio between that of the normal and the regenerating liver, while the tumor supernatant had a ratio 62 per cent higher than the regenerating tissue. Thus, none of the tumor fractions had lower ratios of pentosenucleic acid to protein nitrogen than the normal liver, and two fractions were higher.

SUMMARY

1. Homogenates of regenerating rat livers were separated by differential centrifugation into nuclear, large granule, small granule, and supernatant fluid fractions at various times in the first 8 days and at 23 days after partial hepatectomy. The original homogenate and the fractions were analyzed for protein nitrogen and nucleic acids. The nuclei in the homogenates were enumerated and the data expressed in terms of units per nucleus. Previous results on liver tumors (induced by 4-dimethylaminoazobenzene) were recalculated in terms of units per nucleus for comparison with normal and regenerating livers.

2. There were 131 million nuclei in a gram of normal liver. On the first day of regeneration there was a minimum of 98 million and at 4 days a maximum of 183 million nuclei per gram, and thereafter the number gradually returned to normal.

3. The desoxypentosenucleic acid in the average nucleus increased from 10.0 to 18.3×10^{-12} gm. per nucleus between 12 and 24 hours after partial hepatectomy, and thereafter returned rapidly toward normal as cell division started.

4. In the first day the protein nitrogen of the average cell increased by 17 per cent and then fell to 68 per cent of normal by 4 days. The initial increases occurred in the nuclear and small granule fractions, and later decreases were noted in all the cytoplasmic fractions. By the eighth day all three cytoplasmic fractions contained subnormal amounts of protein nitrogen, but by the 23d day these had all returned to normal levels. The smaller tumor cells contained only 23 per cent as much protein nitrogen as the average normal liver cells, and the distribution in the cell was different from that in either normal or regenerating liver cells.

5. The average liver cell increased its pentosenucleic acid by 62 per cent between 12 and 24 hours after partial hepatectomy, as a result of an approximate doubling of this nucleic acid in the small granule and supernatant fluid fractions. In the period between 2 and 6 days the pentosenucleic acid content of the average cell fell to normal, with the greatest decrease in the same two fractions. The nuclear fraction reached a maximum 3 days after the operation, and the large granules changed relatively little during regeneration. The average tumor cell contained only 36 per cent as much pentosenucleic acid as the average normal liver cell, but the supernatant fluid of these tumor cells had a normal level of this nucleic acid.

6. As a result of increases in pentosenucleic acid during the first 2 days and decreases in protein nitrogen during the next 4 days, the ratio of pentosenucleic acid to protein nitrogen was increased in all fractions during rapid growth. In tumor cells these ratios were normal in the nuclear and small granule fractions, were elevated about

half as much as regenerating liver in the large granules, and exceeded regenerating liver in the supernatant fluid.

7. The advantages of reporting the data in terms of units per nucleus are emphasized.

8. Acidophilic cytoplasmic inclusion bodies were found in all regenerating livers about 1 day after partial hepatectomy; they were no longer observed 2 days after the operation.

REFERENCES

1. Beams, H. W., and King, R. L. The Origin of Binucleate and Large Mononucleate Cells in the Liver of the Rat. Anat. Rec., **83**:281–98, 1942.
2. Boivin, A.; Vendrely, R.; and Vendrely, C. L'acide désoxyribonucléique du noyau cellulaire, dépositaire des caractères héréditaires; arguments d'ordre analytique. Compt. rend. Acad. d. sc., **226**:1061–63, 1948.
3. Brues, A. M.; Drury, D. R.; and Brues, M. C. A Quantitative Study of Cell Growth in Regenerating Liver. Arch. Path., **22**:658–73, 1936.
4. Bucher, N. L. R., and Glinos, A. D. The Effect of Age on Regeneration of Rat Liver. Cancer Research, **10**:324–32, 1950.
5. Bucher, N. L. R.; Scott, J. F.; and Aub, J. C. Regeneration of the Liver in Parabiotic Rats. Cancer Research, **10**:207, 1950.
6. Cain, A. J. An Easily Controlled Method for Staining Mitochondria. Quart. J. Microscop. Sc., **89**:229–31, 1948.
7. Cunningham, L.; Griffin, A. C.; and Luck, J. M. The Desoxyribonucleic Acid Content per Nucleus in Normal, Precancerous, and Cancerous Tissues of the Rat. Cancer Research, **10**:211, 1950.
8. Davidson, J. N., and Leslie, I. A New Approach in the Biochemistry of Growth and Development. Nature, **165**: 49–53, 1950.
9. Dounce, A. L.; Tishkoff, G. H.; Barnett, S. R.; and Freer, R. M. Free Amino Acids and Nucleic Acid Content of Cell Nuclei Isolated by a Modification of Behrens' Technique. J. Gen. Physiol., **33**:629–42, 1950.
10. Gurd, F. N., and Vars, H. M. Pathological Changes after Partial Hepatectomy, with Special Reference to Necrosis in Protein-Depleted Rats. Arch. Path., **48**:140–49, 1949.
11. Guyer, M. F., Animal Micrology, p. 239. Chicago: University of Chicago Press, 1936.
12. Higgins, G. M., and Anderson, R. M. Experimental Pathology of the Liver; Restoration of the Liver of the White Rat Following Partial Surgical Removal. Arch. Path., **12**:186–202, 1931.
13. Landing, B. H.; Seed, J. C.; and Banfield, W. G. The Effects of a Nitrogen Mustard, Tris (2-Chloroethyl) Amine, on Regenerating Rat Liver. Cancer, **2**:1067–74, 1949.
14. Lillie, R. D. Histopathologic Technic, pp. 35, 36, 78. Philadelphia: Blakiston Co., 1948.
15. Mark, D. D., and Ris, H. A Comparison of Desoxyribonucleic Acid Content in Certain Nuclei of Normal Liver and Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**: 727–29, 1949.
16. Miller, E. C.; Miller, J. A.; Sandin, R. B.; and Brown, R. K. The Carcinogenic Activities of Certain Analogues of 2-Acetylaminofluorene in the Rat. Cancer Research, **9**:504–9, 1949.
17. Mirsky, A. E., and Ris, H. Isolated Chromosomes. J. Gen. Physiol., **31**:1–6, 1947.
18. ———. Variable and Constant Components of Chromosomes. Nature, **163**:666–67, 1949.
19. Novikoff, A. B., and Potter, V. R. Biochemical Studies on Regenerating Rat Liver. J. Biol. Chem., **173**:223–32, 1948.
20. Price, J. M.; Miller, E. C.; and Miller, J. A. The Intracellular Distribution of Protein, Nucleic Acids, Riboflavin, and Protein-bound Aminoazo Dye in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. J. Biol. Chem., **173**:345–53, 1948.
21. ———. Intracellular Distribution of Vitamin B_6 in Rat and Mouse Livers and Induced Rat Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**:575–78, 1949.
22. Price, J. M.; Miller, E. C.; Miller, J. A.; and Weber, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. I. 4-Aminoazobenzene, 4-Dimethylaminoazobenzene, 4′-Methyl-, and 3′-Methyl-4-dimethylaminoazobenzene. Cancer Research, **9**:398–402, 1949.
23. ———. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3′-Methyl-, 2′-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene, and 4′-Fluoro-4-dimethylaminoazobenzene. *Ibid.*, **10**: 18–27, 1950.
24. Price, J. M.; Miller, J. A.; Miller, E. C.; and Weber, G. M. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, **9**:96–102, 1949.
25. Schneider, W. C. Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Desoxypentose Nucleic Acid and of Pentose Nucleic Acid. J. Biol. Chem., **161**:293–303, 1945.
26. ———. Phosphorus Compounds in Animal Tissues. II. The Nucleic Acid Content of Homologous Normal and Cancer Tissues. Cancer Research, **5**:717–21, 1945.
27. Schweigert, B. S.; Guthneck, B. T.; Price, J. M.; Miller, J. A.; and Miller, E. C. Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors. Proc. Soc. Exper. Biol. & Med., **72**:495–501, 1949.
28. Stoneburg, C. A. Lipids of the Cell Nuclei. J. Biol. Chem., **129**:189–96, 1939.
29. Stowell, R. E. Nucleic Acids and Cytologic Changes in Regenerating Rat Liver. Arch. Path., **46**:164–78, 1948.
30. ———. Alterations in Nucleic Acids during Hepatoma Formation in Rats Fed *p*-Dimethylaminoazobenzene. Cancer, **2**:121–31, 1949.
31. Sulkin, N. M. A Study of the Nucleus in the Normal and Hyperplastic Liver of the Rat. Am. J. Anat., **73**:107–25, 1943.
32. Umbreit, W. W.; Burris, R. H.; and Stauffer, J. F. Manometric Techniques and Related Methods for the Study of Tissue Metabolism, p. 103. Minneapolis: Burgess Publishing Co., 1945.